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(54) Title: NOVEL METHODS FOR THE IDENTIFICATION OF LIGAND AND TARGET BIOMOLECULES

(57) Abstract

The invention provides for methods for identification of biologically active biomolecules. In one aspect, a biologically active biomolecule such as RNA or a peptide is identified by incorporating random nucleotide sequences in a scaffold constituted by an enzyme activity modulator, transforming substantially identical host cells with the construct obtained thereby and screening the transformed cells ·to identify those where a preselected phenotypic trait has been altered. The randomized DNA is subsequently isolated from the phenotypically altered cells and the peptide and/or RNA encoded by the random sequence is determined. In turn, interaction partners which are putative drug targets are identified and isolated by use of the peptide and/or RNA as part of affinity reagents. A preferred scaffold is derived from the potato inhibitor I family of protease inhibitors and exemplified is the barley chymotrypsin inhibitor 2A (CI–2A). Another aspect relates to the identification of novel enzyme inhibitors by using substantially the same approach, but screening specifically for changes in target enzyme activity. Also disclosed are methods of producing the relevant transformation and expression vectors as well as methods for identifying lead compounds and drug targets for use in drug development. Finally, the invention also includes within its scope a method for the preparation of a medicinal product.

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NOVEL METHODS FOR THE IDENTIFICATION OF LIGAND AND TARGET BIOMOLECULES

FIELD OF THE INVENTION

The present invention pertains to a novel method for the
identification/preparation of peptides or ribonucleic acids
5 capable of modulating the activity *in vivo* of target enzymes
in eukaryotic cells. More specifically, the invention provides
a method for identification/preparation of hitherto unknown
enhancers as well as inhibitors of *in vivo* enzyme activity in
eukaryotic cells. Furthermore, the invention relates to me-
10 thods for identification of unknown interactions (*i.e.* identi-
fication of a target and/or a ligand but also of hitherto
unknown interactions between known ligands and known targets).
These novel methods employ enzyme inhibitor structures as
scaffolds in order to intracellularly display potentially
15 biologically active peptides or ribonucleic acids in a stable
form. Also disclosed herein are methods for the preparation of
the hitherto unknown ligands or targets as well as methods for
the preparation of vectors and transformed cells carrying the
genetic information encoding these ligands and targets. Fi-
20 nally, the invention relates to a method for the preparation
of a medicinal product which is based on initial identifica-
tion of targets or ligands according to the present invention.

BACKGROUND OF THE INVENTION

The CellScreen™ technique is a method which allows for the
25 identification of peptide sequences having biological activity
in vivo and which is disclosed in WO 96/38553. In short,
libraries of random peptides are expressed intracellularly in
eukaryotic (eg. mammalian) cells, such that one cell expresses
one single or a few heterologous short peptides. Cells that
30 change a preselected phenotype under certain conditions can be

isolated and the peptide that they express can hence be iden-
tified. The intracellular component with which the peptide
interacts (the target molecule) may subsequently be obtained
using e.g. affinity columns carrying the immobilized synthetic
5 peptide.

Although the CellScreen™ technology has shown great promise
for identifying new drug targets, it is an inherent problem
that the intracellular environment is relatively hostile to
many heterologous expression products. In other words, inter-
10 esting peptide or nucleic acid sequences which potentially are
capable of interacting with an important target molecule may
be degraded or inactivated inside the cell before any effect
on phenotype can be detected.

OBJECT OF THE INVENTION

15 It is an object of the invention to provide improvements in
the CellScreen™ technology by overcoming the above-mentioned
problems of potential instability of expressed sequences.
Furthermore, it is an object of the invention to expand the
utility of the CellScreen™ technology to also encompass
20 screening in prokaryotic cells.

SUMMARY OF THE INVENTION

A significant number of enzyme activity modulators of plant,
microbial and eukaryotic cell origin have been described, cf.
below.

25 Since many of the naturally occurring processes inside cells
are regulated by enzymes, the inventors disclose herein a
method for expression of large intracellular libraries of such

enzyme activity modulators in which the active site of said
enzyme activity modulators have been altered by introduction
of stretches of randomized amino acid sequences or by intro-
duction of random nucleotides at specific sites in the active

5 site. This creates libraries of putative modulators capable of
modulating the activity of an array of different enzymes
inside cells. By expressing these modulators in cell lines
according to a novel variation of the CellScreen™ technology
the enzymatic regulatory mechanisms inside the individual cell

10 in said cell line will be affected differently leading to
different phenotypic properties such as e.g. resistance to-
wards hypoglycemia, cytokine killing, toxic compounds, virus
infection etc.

The main advantage of using known enzyme activity modulators

15 such as enzyme *inhibitors* as scaffolds is that many of these
in their native form are stable in the intracellular environ-
ment. The problem of using e.g. antibody fragments as scaf-
folds for intracellular presentation of random peptides is
that many such antibody fragments are susceptible to the

20 proteolytic and reducing intracellular environment and there-
fore are unsuitable as intracellular scaffolds. On the other
hand, enzyme activity modulators can, if carefully tailored,
maintain their intracellular stability and at the same time
incorporate random sequences which are screened for biological

25 activity. Furthermore, the effectivity of such a screen for
biologically active substances will be higher than if using an
unstable scaffold (such as a e.g. a coiled coil structure) or
no scaffold at all, since none or only a very limited number
of the randomized sequences will be degraded before they can

30 exert their effects in the cells. Finally, a majority of such
enzyme activity modulators have an active site which is the
perfect position in the molecule to modify, since the active
site is normally presented in a stable configuration to the
environment.

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Hence, one aspect of the invention pertains to a method
for identifying an *in vivo* active modulator of activity of a
target enzyme, the method comprising the steps of (a) pre-
paring a pool of expression vectors, each vector of said pool
5   containing at least one member from a library of randomly
modified nucleotide sequences derived from a parent nucleotide
sequence encoding a parent peptide or parent ribonucleic acid
which modulates the target enzyme activity, (b) transforming a
population of substantially identical cells with said vectors
10 of said pool so as to obtain transformed cells, said substan-
tially identical cells being ones which harbour the target
enzyme, (c) culturing said transformed cells under conditions
facilitating expression of said randomly modified nucleotide
sequences, (d) examining said transformed cells and isolating
15 transformed cell(s) wherein the activity of the target enzyme
is modulated, and (e) identifying the modulator by determining
said randomly modified nucleotide sequence of said vector
present in cell(s) isolated in step (d) and/or determining the
amino acid sequence or the ribonucleic acid sequence of the
20 expression product encoded by said randomly modified nucleo-
tide sequence.

In this aspect of the invention it is normally preferred that
the randomly modified nucleotide sequences consist of 1) an
invariable part of the parent nucleotide sequence, and 2)
25 random nucleotides. In line with the above, the invariable
part of the parent nucleotide sequence preferably encodes a
scaffold portion of the parent peptide or of the parent ribo-
nucleic acid which serves to stabilize said polypeptide frag-
ment or ribonucleic acid fragment.

30 As mentioned above, the use of enzyme modulator scaffolds also
provides for expression of *stable*, biologically active modula-
tors which interact with other biomolecules than enzymes. In
other words, in cases where random nucleotides are inserted in

a scaffold structure, the outcome will be a stable expression
product, the activity of which does not necessarily have any-
thing to do with enzyme activity modulation.

Hence, another part of the invention is a method for identify-
5 ing a modulator in the form of a biologically active polypep-
tide fragment or ribonucleic acid fragment which is capable of
detectably modulating, *in vivo*, a phenotypic trait in a cell,
the method comprising the steps of

(a) preparing a pool of expression vectors, each vector of
10 said pool containing at least one member from a library of
randomly modified nucleotide sequences derived from a parent
nucleotide sequence encoding a parent peptide or parent ribo-
nucleic acid which *in vivo* modulates activity of a known
enzyme, wherein the randomly modified nucleotide sequences
15 comprise
    –   an invariable part encoding a scaffold portion of the
         parent peptide or of the parent ribonucleic acid, said
         scaffold portion serving to stabilize said polypeptide
         fragment or ribonucleic acid fragment, and
20 –   random nucleotides,
    (b) transforming a population of substantially identical cells
    with said vectors of said pool so as to obtain transformed
    cells, (c) culturing said transformed cells under conditions
    facilitating expression of said randomly modified nucleotide
25 sequences, (d) examining said transformed cells and isolating
    transformed cell(s) wherein the preselected phenotypic trait
    is modulated by the presence of the expressed randomly modi-
    fied nucleotide sequence, and (e) identifying the modulator by
    determining said randomly modified nucleotide sequence of said
30 vector present in cell(s) isolated in step (d) and/or deter-
    mining the amino acid sequence or the ribonucleic acid se-
    quence of the expression product encoded by said randomly
    modified nucleotide sequence.

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Finally, the invention also pertains to the general use of
intracellularly stable scaffold proteins, ribonucleotides, or
fragments thereof for the presentation of random sequences in
the CellScreen™ technology. As mentioned above, although the
5 concept of using scaffold molecules has been discussed in the
prior art, the issue of the *stability* of the scaffold system
has not been detailed.

The stability and usefulness of a putative intracellular scaf-
fold is dependent on a number of factors. First of all, it is
10 essential that the relevant cell wherein the scaffold is to be
expressed is capable of expressing the scaffold molecule in a
functional form; that is, in prokaryotic systems some eukaryo-
tic proteins will not fold correctly, hence rendering the use
of such a protein unsuitable as a scaffold in that type of
15 cell. Second, the scaffold should be relatively resistant to
the reducing and catalytic environment inside intact cells.
However, even when a scaffold molecule is relatively suscep-
tible to the inactivating nature of the intracellular environ-
ment, this can be remedied if the production rate of the
20 scaffold molecule is sufficiently high.

In steady state, the intracellular concentration of a scaffold
molecule will be a function of the following formula:

$$C_{scaffold} = \frac{R_p}{R_d}$$

-where R_p is the rate of production of the scaffold molecule
25 (moles $\cdot$ s^{-1}) and R_d is the inactivation constant for the scaf-
fold molecule (l $\cdot$ s^{-1}), *i.e.* the rate of inactivation of the

scaffold molecule is determined by $\frac{\delta M}{\delta t} = R_d C_{scaffold}$ (which, in

steady state, of course equals R_p. In other words, when assess-
ing the suitability of a potential scaffold molecule, it
30 should according to the present invention be tested whether
the molecule can be kept at a sufficiently high concentration

inside the relevant cell wherein the CellScreen™ test is going
to be carried out.

Therefore, a very broad aspect of the invention pertains to a
method for identifying a modulator in the form of a biolo-
5   gically active polypeptide fragment or ribonucleic acid frag-
ment which is capable of detectably modulating, *in vivo*, a
phenotypic trait of a cell, the method comprising the steps of
(a) preparing a pool of expression vectors, each vector of
said pool containing at least one member from a library of
10   randomly modified nucleotide sequences derived from a parent
nucleotide sequence encoding a parent peptide or parent ribo-
nucleic acid which is stable intracellularly, wherein the
randomly modified nucleotide sequences comprise
-       an invariable part encoding a scaffold portion of the
15   parent peptide or of the parent ribonucleic acid, said
scaffold portion serving to stabilize said polypeptide
fragment or ribonucleic acid fragment, and
-       random nucleotides,
(b) transforming a population of substantially identical cells
20   with said vectors of said pool so as to obtain transformed
cells, (c) culturing said transformed cells under conditions
facilitating expression of said randomly modified nucleotide
sequences, (d) examining said transformed cells and isolating
transformed cell(s) wherein the preselected phenotypic trait
25   is modulated by the presence of the expressed randomly modi-
fied nucleotide sequence, and (e) identifying the modulator by
determining said randomly modified nucleotide sequence of said
vector present in cell(s) isolated in step (d) and/or deter-
mining the amino acid sequence or the ribonucleic acid se-
30   quence of the expression product encoded by said randomly
modified nucleotide sequence. In this aspect of the invention,
the expression product of the nucleic acid sequence which
encodes the intracellularly stable parent peptide or parent
ribonucleic acid is one which, when produced by the substan-

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tially identical cells, is present in an effective concentra-
tion and in a functional state.

In other words, it is essential that the suitability of the
scaffold molecule is evaluated prior to performing the steps
5 of the CellScreen™ technology in order to confirm that the
scaffold molecule in unmodified form can be expressed and
maintained at a sufficiently high concentration/activity in
the cellular system where the method of the invention is to be
exercised.

10 According to WO 96/38553, the isolation of a drug target mole-
cule can be made more efficient if the random peptide sequen-
ces are inserted into larger polypeptides functioning as
scaffolds for display of the random amino acid sequences. Such
scaffolds would probably also lead to higher affinity interac-
15 tion with the target molecule.

Nothing is, however, mentioned about the use of scaffolds de-
rived from naturally occurring protein inhibitors of enzymes.
Inhibition of enzymatic activity by such inhibitors – as
opposed to the simple binding of a target protein inside a
20 cell as was suggested in the CellScreen™ technology – is a
much more efficient way to affect intracellular biochemical
events.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

25 In the following, a number of terms will be defined for the
purposes of the present disclosure:

A "modulator" is in the present context a biomolecule which, when expressed *in vivo,* effects the activity of another biomolecule in the cell. Thus, the modulator in essence can inhibit or enhance the activity of the biomolecule. Furthermore, the

5   modulator can interact directly with the biomolecule, but the effect might as well be indirect, *i.e.* the activity change of the biomolecule is brought about by changes in the cell's biochemical machinery, changes which are ultimately the result of the presence of the expression product of the randomized

10   nucleic acid sequence.

A "randomly modified nucleotide sequence" is a nucleotide sequence which in a number of positions has been subjected to insertion or substitution by nucleotides, the nature of which cannot be predicted. In many cases the random nucleotides or

15   nucleotide sequences inserted will be "completely random" (e.g. as a consequence of randomized synthesis or PCR-mediated mutagenesis). However, as will appear from the disclosure below, the random sequences can also include sequences which have a common functional feature (e.g. reactivity with a

20   ligand of the expression product) or the random sequences can be random in the sense that the ultimate expression product is of completely random sequence with e.g. an even distribution of the different amino acids.

"Substantially identical cells" is a term herein intended to

25   designate cells which all exhibit a specific phenotypic trait in such a manner that a change in the expression of said trait in one cell due to an interaction effected by the introduction of random nucleotides according to the inventive methods would also occur in one of the other substantially identical cells

30   had these been transformed with the same vector. In other words, the important parameter to assess when choosing substantially identical cells in the inventive methods is whether an observed change in one cell's exhibition of the phenotypic

trait can be taken as an indication that any other cell in the
population would have behaved the same way as a consequence of
the same change. Hence, substantially identical cells can for
instance be clonal cells or cells of a cell line or they can
5   be cells of a cell culture or a tissue culture.

A "phenotypic trait" is the observable result of a certain
gene composition in a cell (genotype), i.e. a property of a
cell (detected by chemical, physical, immunological or any
other suitable means) which depends on the presence of one or
10 several genes and the expression rate thereof. Thus, the
phenotypic trait can be any of a number of different proper-
ties: activity of an enzyme, effects of interaction between
receptors and ligands, cell survival rate, presence or absence
of an antigen, expression rate, etc.

15 "Peptide" is in the present context intended to mean both
short peptides of from 2 to 10 amino acid residues, oligopep-
tides of from 11 to 100 amino acid residues, and polypeptides
of more than 100 amino acid residues. Furthermore, the term is
also intended to include proteins, i.e. functional biomole-
20 cules comprising at least one polypeptide; when comprising at
least two polypeptides, these may form complexes, be cova-
lently linked, or may be non-covalently linked. The polypep-
tide(s) in a protein can be glycosylated and/or lipidated
and/or comprise prosthetic groups.

25 When using the term "biologically active" to designate a
molecule is herein meant that the molecule in question exhi-
bits a detectable effect on living cells, i.e. that the mole-
cule interacts with the biology of the living cell so as to
produce an effect which can be recognized as a change in the
30 cell's phenotype.

"*In vivo*" is herein mean to designate the environmental condi-
tions inside living cells (*i.e.* cells which are metabolically
active and can maintain their vital functions); the living
cells may be kept in culture or may be present in a natural
5   habitat (e.g. functioning as part of a larger, multicellular
organism). Thus, the term "*in vivo*" also refers to *in vitro*
culturing of cells as long as the effect being observed is
taking place in the living cell.

"Transformation": A process by which the genetic material car-
10 ried by an individual cell is altered by incorporation of exo-
genous DNA into its genome.

"Transfection": The uptake, incorporation, and expression of
recombinant DNA by cells.

"Transduction": The transfer of genetic information from one
15 cell to another by way of a viral vector.

The term "effective part" when used in the context of a pro-
tein, peptide, or ribonucleic acid is in the present context
intended to mean a part (e.g. a subsequence or, in the case of
an n-meric protein, a less-than-n-meric molecule) which has
20 retained the desired functionality of the native molecule from
which the protein or peptide is derived. For instance, in the
case of CI-2A only the truncated form of the molecule seems to
be necessary to ensure expression of the active inhibitor
intracellularly, and hence the truncated form of that molecule
25 constitutes an effective part of CI-2A; cf. also the Examples
herein.

Unless otherwise indicated, nucleotide sequences are presented
herein in the 5'-3' direction and amino acid sequences are
presented so as to set out with the N-terminus at the left.

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<u>General properties of the inventive method</u>

In general, the disclosures in WO 96/38553 and WO 97/27212 relating

5   to preparation of randomized sequences, to the choice of
       fusion partners (except for the choice of scaffolds) for
       the random sequences, to the choice and composition of
       targeting sequences, to the choice of nucleic acids to be
       randomly modified, to the choice of randomization method,
       to the methods of introducing the nucleic acids into the
10   relevant cell type, to the choice of (retroviral) vectors
       (where applicable), to the method of producing the vec-
       tors, the choice of promoters, to the choice of packaging
       cells (where applicable), to the methods of concentrating
       infectious virions from the packaging cells (where appli-
15   cable), to the choice of substantially identical cells to
       use in the method, to the type of phenotypic changes
       detected, to the manner in which the change is detected,
       to methods of isolating the phenotypically changed cells,
       to the isolation and sequence determination of the ran-
20   domly modified sequences, to the isolation and character-
       ization of the target for the randomized product, to
       screening methods, and to the choice of applications of
       the methods

       also are relevant for the purposes of the present invention.
25 Therefore, the disclosures of these two patent applications
   are hereby incorporated by reference herein. However, since
   both of these references are focussed on the use of the gen-
   eral principle in higher eukaryotic cells, the present disclo-
   sure will also detail on embodiments pertaining to the use in
30 prokaryotic systems, cf. below. However, the more general part
   of the two above-referenced disclosures which without any
   difficulty for the skilled person could be applied in the

context of a prokaryotic system are also regarded as relevant
and important embodiments of the present invention insofar as
it relates to the use of the methods in prokaryotic systems.

5   It is normally preferred that the transformed cells which are
being examined in step (d) predominantly carries (and expres-
ses) one single copy of the vector. By ensuring this, the
interpretation of a change in phenotype of the cells becomes a
much easier task, whereas the interpretation of a phenotype
change in cells expressing more than one single randomized
10   sequence renders unclear which of the transforming vectors is
responsible for the change.

To ensure that predominantly one vector has transformed each
of the cells examined it is e.g. feasible that the transforma-
tion step (b) is performed under such conditions that the
15   cells transformed are predominantly or at most transformed
with one single vector from said pool (this can e.g. be
achieved by adjusting the concentration of infectious virions
in embodiments of the invention where the transformation is
obtained by means of transduction), or wherein, prior to
20   carrying out step (d), cells being transformed with more than
one vector from said pool are substantially excluded from the
further steps. This latter option requires that it is possible
to quantify the number of transforming vectors and this can be
achieved by including a detectable marker in the expression
25   product, e.g. a flourescent probe. Another option is to re-
screen cells which exhibit changes in phenotype, thereby
ascertaining whether more than one vector has transformed the
cell.

It will be understood that the molecule chosen for the purpose
30   of being a scaffold, and wherein the random sequences are
ultimately introduced, can be either a peptide sequence or a
nucleic acid sequence, such as an RNA fragment interfering (in

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an antisense-manner) with mRNA, tRNA or with a ribozyme. Alternatively, such an RNA fragment could exhibit ribozyme activity itself, thereby having an indirect influence on the expression rate of other enzymes. At any rate, the resulting
5   product, *i.e.* the randomized expression product, can be a peptide or a ribonucleic acid such as a ribozyme.

One important feature of the scaffold is, as mentioned above, that it is stable towards proteolytic attack and/or is insensitive to a reducing environment, such as the one which
10 is found intracellularly.

In preferred embodiments of the invention the random nucleotides are introduced in part(s) of the parent nucleotide sequence which encode(s) the active site(s) of the parent peptide or parent ribonucleic acid, or the part(s) which
15 encode(s) structure(s) interfering with the active site(s). As discussed above, the active site (as well as other exposed structures of the scaffold) need to be stably presented to the environment in order to be able to interact with other biomolecules. Hence, preferably the *invariable* part of the
20 nucleotide sequence encodes truncated parts of the parent peptide or parent ribonucleic acid sufficient to maintain stability of the randomized product.

In some embodiments it is preferred that the invariable part of the parent nucleotide sequence encodes a peptide which is
25 free from disulfide bridges. This is due to the fact that disulfide bridges are not formed in the nucleus or in the cytosol. Hence, in cases were the scaffold must be in a functional state when present in the nucleus or the cytosol, it would normally be preferable to use a scaffold which does not
30 contain disulfide bridges or which do not rely on these in order to maintain stability and functionality. On the other hand, in embodiments where it is desired that the randomized

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expression product is confined to the ER, or to another com-
partment allowing for the presence of stable disulfide brid-
ges, before the randomized sequence is presented to the envi-
ronment in a satisfactory manner, it is of course desirable

5 that the invariable part of the parent nucleotide sequence
encodes a peptide having disulfide bridges, because the chan-
ces of having a correctly folded and functional scaffold
outside such compartments is relatively small.

It will be understood that the random nucleotides are prefer-
10 ably introduced in the form of an insertion or a substitution
into the parent nucleotide sequence, optionally in combination
with deletion(s) in the parent nucleotide sequence. Deleted
sequences in the parent polypeptide could e.g. be parts of an
active site, the presence of which in unaltered form is toxic
15 or otherwise deleterious to the transformed cells.

The number of random nucleotides introduced can vary to a
great degree but normally the number is between 3 and about
100. In this range it is preferred that at least 5, such as at
least 7, and better, at least 9-12 random nucleotides are
20 introduces. On the other hand, it is preferred that at most
90, such as at most 70 or 80 random nucleotides are intro-
duced. The most preferred number of introduced randomized
nucleotides varies between 15-60, preferably 20-55 or 25-50
nucleotides. Especially preferred numbers of random nucleo-
25 tides are 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30,
31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45,
46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, and 60
nucleotides.

The random nucleotides are introduced in the scaffold in the
30 form of nucleotide sequences and/or in the form of single
random nucleotides introduced at specific sites in the parent
nucleotide sequence. A variation is to substitute a part of

the scaffold sequence with a sequence which retains parts of
the scaffold sequence (e.g. those which are essential for
stability/functionality) but where other parts are randomized.

The random nucleotides are preferably selected from the group
5 consisting of
   -    synthetic, completely random deoxyribonucleotides;
   -    synthetic random DNA sequences, wherein limitation on
        randomization of some nucleotides is introduced so as to
        limit the number of available sequences and/or to avoid
10   undesired stop codons and/or to facilitate introduction
        of post-translational modifications of expressed pepti-
        de(s);
   -    synthetic random DNA sequences as in (1) or (2) coupled
        to a sequence encoding a purification tag; and
15 -   CDR encoding nucleotide sequences isolated from a library
        of immune-competent cells raised against an antigen (in
        this embodiment it is preferred that CDR encoding nucleo-
        tide sequences encode CDR-3 peptide sequences).

The latter type of "randomization" actually introduces a re-
20 striction on randomness which ensures that the sequences
introduced encodes an antigen recognizing region. It is,
however, well known that a polyclonal immune response against
an antigen consist of a large number of immune competent cells
which all react with the same antigen (or perhaps even with
25 the same epitope) but the correlation between amino acid
sequences of the CDRs and recognition of the epitope(s) is
virtually impossible to deduce.

An alternative way to introduce limitations on the randomness
of the nucleic acid sequences which are ultimately tested in
30 the substantially identical cells is the following: Upon
preparation of the vectors, they are used in a 1st round of
phage display, where the phages transformed with the vectors

are panned against a library containing a ligand of choice. As
for the technique of employing CDR encoding sequences, the
result is that the sequences which are ultimately tested in
the substantially identical cells are "unpredictable" (and
5   thereby random) but nevertheless selected on the basis of a
functional feature. Again, the lack of known correlation
between nucleic acid sequences and the interaction in three-
dimensional space between the expression product and a ligand
of choice has the consequense that the tested subgroup of
10 sequences still is randomized.

In a special embodiment of the above-technique where the
method of the invention is combined with phage display, both
test systems are repeated in an alternating manner, that is a
shuffling between intracellular expression in the substantial
15 identical cells and panning of a phage library.

In order to obtain an controlled distribution of amino acids
in the randomized peptides, when the modulator is a peptide,
it is practical that the random nucleotides are prepared by
random codon synthesis where defined DNA codons are synthe-
20 sized in a random order; a thorough description of this prin-
ciple is given in WO 96/38553, cf. Example 1 therein. The
preferred embodiment in this context is one wherein the rela-
tive amount of synthesized codons ensure that all encoded
amino acids will be present with substantially the same fre-
25 quency in the total of encoded polypeptide fragments, i.e.
that the chance of encountering one specific amino acid in a
library peptide is substantially the same as for any other
encoded amino acid.

In order to introduce the randomized fragments properly into
30 the vectors, it is according to the invention preferred that
the random nucleotides are introduced into the expression
vector by the principle of site directed PCR-mediated mutagen-

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esis. However, other options are known to the skilled person, and it is e.g. possible to insert synthetic random sequence libraries into the vectors as well.

Apart from having the randomized fragment of the expression
5 product introduced into a scaffold in accordance with the pre-
sent invention, it is often necessary to couple the random sequence to a fusion partner by having the randomized nucleo-tide sequence fused to a nucleotide sequence encoding at least one fusion partner. Such a fusion partner can e.g. facilitate
10 expression and/or purification/isolation and/or further stabilization of the expression product.

For the purposes of purification, the fusion partner can include a purification tag such as His_6 tag, myc tag, BSP biotinylation target sequence, of BirA, flu tag, lacZ, and
15 GST. Furthermore, the fusion partner may include a sorting signal or a targeting sequence, cf. the discussions below.

In embodiments where the modulator is itself a modulator of enzyme activity, it is in theory possible to effect both the K_M and/or the V_{max} of the relevant enzyme. A reduction in K_M re-
20 sults in less effectivity of the relevant enzyme insofar that an increased substrate concentration is required to obtain 50% of maximum activity of the enzyme. An increase of K_M has the opposite effect. Of course, interference with an enzyme which effects V_{max} has as a consequence that the maximum possible rate
25 of activity of the enzyme is increased (when V_{max} is increased) or decreased (when V_{max} is increased). At any rate, the modula-tor of the enzyme activity will give the phenotypic impression that the enzyme activity has either been inhibited or stimu-lated. It is preferred in this embodiment that the modulator
30 is an inhibitor.

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When the method of the invention has finally lead to the identification of a modulator or of a target therefor, it is preferred that the 3-dimensional structure of the identified modulator is resolved, since this allows for the implementa-

5 tion of rational drug screening and computer drug modelling methods.

Use of the inventive methods in prokaryotic systems

The originally envisaged technology disclosed in WO 96/38553 focussed on screening for interactions in *eukaryotic* cells.

10 However, the technology is also applicable in prokaryotic systems.

For instance, it is expected that the present invention will allow for identification of hitherto unknown interactions in pathogenic bacteria, interactions which will be useful in the

15 course of developing new antibiotics. Since the inventive methods allows for the identification of both novel ligand peptides and ribonucleic acids as well as of the target molecules for these ligands, the investigator is provided with the necessary tools for instigating computer drug modelling and

20 for performing traditional drug screening, once such ligands and/or targets have been identified/isolated.

However, apart from the approach of identifying antibacterial effects and substances, the method also opens up for improvements in industrial fermentation processes. In such cases it

25 will e.g. be possible to identify biomolecules which are important in the biochemical pathways in lactic acid bacteria and thereby provide tools for the production of new dairy products such as cheese, yoghourt, and other products of lactic acid bacterial fermentation.

Somewhat related to this approach is the use of the methods of
the present invention in screening performed on bacterial cul-
tures used in purification processes. It is well-known in the
art of e.g. waste water purification that the microbiological

5   cultures (activated sludge) which conduct the degradation of
organic material, are relatively vulnerable *vis à vis* changes
in the environment and therefore the provision of more robust
strains of bacteria would be one way to improve such systems.
Alternatively, the method of the present invention would also

10 allow for the identification/isolation of ligands and targets
in such bacteria which, when interacted with, can lead to e.g.
increased efficacy in degradation of specific organic or inor-
ganic substrates.

As will be appreciated from the above, the present invention
15 therefore is highly useful in prokaryotic systems.

For the purposes of using the method of the invention in
prokaryotic cells, it is preferred that the prokaryotic cells
are bacteria selected from the group consisting of *Bacillus*
spp. (e.g. *B. anthracis*, *B. subtilis* and *B. cereus*),

20 *Clostridium* spp. (e.g. *C. botulinum*, *C. difficile*, *C. perfrin-
gens*, and *C. tetani*), *Corynebacterium* spp. (e.g. *C. diphthe-
riae*, and *C. pyogenes*), *Staphylococcus* spp. (e.g. *S. aureus*
and *S. albicans*), *Streptococcus* spp. (e.g. *S. pneumoniae*, *S.
pyogenes*, and *S. agalactiae*), *Escherichia coli*, *Serratia*

25 *marcescens*, *Klebsiella* spp. (e.g. *K. pneumoniae*), *Proteus* spp.
(e.g. *P. mirabilis*), *Citrobacter* spp. (e.g. *Citrobacter freun-
dii*), *Salmonella* spp. (e.g. *S. typhi*, *S. typhimurium*, *S.
shottmülleri* and *S. paratyphi*), *Shigella* spp. (e.g. *S.
dystenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*), *Pseudo-

30 monas* spp. (e.g. *P. aeruginosa*, *P. pseudomallei*, and *P. mal-
lei*), *Acinetobacter* spp., *Aeromonas* spp., *Plesiomonas* spp.,
Yersinia spp. (e.g. *Y. pestis*, *Y. enterocolitica*, and *Y.
pseudotuberculosis*), *Francisella tularensis*, *Vibrio* spp. (e.g.

V. cholerae and *V. parahaemolyticus)*, *Campylobacter* spp. (e.g. *C. jejuni* and *C. coli)*, *Helicobacter pylori*, *Haemophilus* spp. (e.g. *H. influenzea*, *H. parainfluenzae*, and *H. aegyptius)*, *Bordetella* spp. (e.g. *B. pertussis*, *B. parapertussis*, and *B.*

5 *bronchiseptica)*, *Brucella* spp., *Neisseria* spp. (e.g. *N. gonno-rhoeae* and *N. meningitidis)*, *Treponema pallidum*, *Leptospira interrogans; Borrelia* spp. (e.g. *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, and *B. recurrentis)*, *Legionella pneumophila*, *Listeria monocytogenes*, *Mycobacterium* spp. (e.g.

10 *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. kansasii*, and *M. leprae)*, *Treponema pallidum*, *Chlamydia trachomatis*, *Actino-myces* spp., *Rickettsia* spp., and *Mycoplasma* spp. (e.g. *M. pneumoniae)*.

This list of bacteria thus entails bacterial families and spe-
15 cies which are involved in pathology of a large number of dis-
eases in humans. Of these, *E. coli* and *B. subtilis* are also
used for industrial fermentation; this is also the case for
lactic acid bacteria, notably *Lactococcus* spp. and *Lactobacil-lus* spp. and therefore it is also preferred that the inventive
20 methods, when employed in bacterial systems, are performed on
such non-pathogenic species.

When using the inventive methods in a prokaryotic system, it
is very often interesting to identify those cells which are
impaired in growth or lethally damaged due to the presence of
25 the heterologous expression product introduced according to
the invention. This, however, is not completely unproblematic
since the main phenotypic trait associated with e.g. bacterial
death is *absence* of the bacterium.

It is therefore necessary to device an experimental setup
30 which will allow identification of cells transformed so as to
be less good survivors than other otherwise corresponding
cells. One advantage is that expression of the heterologous

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genetic material is under the control of an inducible pro-
moter. In this way it is possible to expand colonies of cells
which have been transformed with genetic material which, when
expressed, is lethal or growth-impairing to the cells. After
5 that, the expression can be switched on and careful examina-
tion of expanded colonies should reveal those clonal colonies
which do not follow the same growth pattern as e.g. an un-
transformed control.

One method of doing this is to spread transformed cells on
10 plates with growth medium and allow the cells to grow up to a
pre-determined average size. The spreading of cells should be
such that the visible colonies forming will generally be com-
prised on one single clone of cells. When the pre-determined
size of colonies have been reached, all plates are blotted to
15 a carrier medium so as to prepare a "negative" of each agar
plate. After this, the expression of the inserted random
sequences is induced and the colonies are allowed to grow
again. The plates are examined continuously or at suitable
intervals (e.g. by means of digital image processing systems
20 well known to the skilled person). Those colonies which reveal
an impaired or arrested growth compared to the remainder of
the colonies or compared to controls are thereafter identi-
fied, since the growth pattern of each colony in an automated
manner can be followed. These colonies can then be identified
25 and isolated from the "negative" blot and it is thereafter a
relatively simple procedure to extract the transforming ge-
netic material and determine the sequence thereof.

In this context, one interesting option is to render
antibiotic-resistant bacteria non-resistant. The growth medium
30 either contains, or is during culturing enriched with, the
antibiotic in question and the colonies which upon induction
of expression can be demonstrated to be less drug resistant
than controls are examined further. Also pure

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bactericidal/bacteriostatic effects can be examined. In such
an embodiment, the bacteria are e.g. cultured on a suitable
growth medium. Those colonies which after induction of expres-
sion shows evidence of reduced or arrested growth are examined
5 further: It is expected that some of the bacterial cells will
be demonstrated to carry genetic material encoding an expres-
sion product which interacts with novel (or known) targets for
antibacterial agents.

Another phenotypic trait of interest is of course superior
10 *survival* of cells. It is, when dealing with utilisable bacte-
ria, of interest to identify targets which will increase the
survival rate of the bacteria. For example, bacteria used in
industrial fermentations normally can be lethally damaged as a
consequence of their own uncontrolled production of heterolo-
15 gous expression products. If genes or target molecules can be
identified which have a positive effect on the survival of
such bacteria, the economic potential is enormous, since a
fermentation process will be rendered more economic (less need
to startup of new fermentations). Similarly, bacteria used in
20 e.g. waste water purification can be made more resistant
against toxic agents in their environment.

The experimental setup in this context is relatively simple:
The transformed bacteria are simply subjected to the poten-
tially lethal condition, and only colonies which exhibit a
25 superior survival are isolated and examined (and that will
typically be the colonies which are detectable). A setup like
the above-described for identifying cell death should thus not
be necessary.

Finally, a large group of phenotypic traits to be examined are
30 those which can be detected by e.g. biochemical or immunologi-
cal means. It is expected that the method of the invention
will allow for identification of systems in bacterial cells

which, when properly modulated, can render the bacteria more effective as producers in industrial fermentation. Such phenotypic traits could e.g. be changes in enzyme activity, changes in receptor density, changes in expression rate etc.

5 Special considerations apply when the randomized expression product is fused to a fusion partner which decides the final location of the expression product. Signal sequences in prokaryotes are well-known in the art, but it should briefly be mentioned that membrane-anchoring signals are known, and it

10 is also possible to export the expression product to the periplasmic space of bacteria. Finally, it is also possible to include secretion signals so as to allow the isolation of the expression product from culture supernatant. However, in many cases it is of course most relevant to keep the expression

15 product inside the prokaryotic cytoplasm.

<u>Use of the method in eukaryotic systems</u>

It is especially preferred that the inventive method utilises eukaryotic cells as the substantially identical cells in order to allow screening for active biomolecules. Hence, these

20 eukaryotic cells can be fungal cells, protozoan cells, animal cells, and plant cells.

As is the case for bacteria, a number of fungi are pathogens in mammals, and therefore the present technology will, in parallel with what has been described above concerning anti-

25 bacterial agents, be useful for identifying antifungal agents by using pathogenic fungi as the substantial identical cells in the method. Furthermore, fungi (especially yeast strains), like bacteria, are also utilised in fermentation processes (e.g. in the wine and brewing industries), and the method can

30 therefore also be utilised using such non-pathogenic fungi as the substantially identical cells which are transformed with

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the vectors, whereby improvements in these strains can be
obtained.

Preferred examples of fungi serving as the eukaryotic cell in
the inventive methods are *Epidermophyton* spp., *Trichophyton*
5   spp., *Microsporum* spp., *Candida albicans*, *Philophora* spp.,
Coccidioides immitis, *Histoplasma capsulatum*, *Blastomyces
dermatitidis*, *Paracoccidioides brasiliensis*, *Cryptococcus
neoformans*, *Aspergillus* spp., *Saccharomyces cerevisiae*,
Klyveromyces lactis, and *Piccia pastoris*.

10 Unlike fungal cells, protozoan cells are only relevant as
pathogens for humans and other mammals, although some protozo-
ans form part of biocultures conducting biological waste water
purification. The method of the invention is therefore contem-
plated to be useful in identifying new targets for
15 antiprotozoan agents. In this context, the preferred protozoan
cells used as the substantially identical cell in the methods
of the invention are selected from the group consisting of
Giardia lamblia, *Trichomonas vaginalis*, *Dientamoeba fragilis*,
Trypanosoma spp., *Leishmania* spp., *Entamoeba histolytica*,
20 *Naegleria fowleri*, *Acanthamoeba castellani*, *Harmanella* spp.,
Isospora belli, *Cryptosporidium* spp., *Sarcocystis* spp.,
Toxoplasma gondii, *Plasmodium* spp. (e.g. *P. falciparum*, *P.
vivax*, *P. malariae*, *P. knowlesi*, and *P. ovale*), *Babesia* spp.,
and *Balantidium coli*.

25 Also plant cells are according to the invention interesting as
target eukaryotic cells. The plant cells can be any plant cell
which can be subjected to genetic engineering techniques
allowing for single cell expression and growth. Thus, cells
derived from e.g. *Nicotiana tabacum* (tobacco plant), *Arabidop-*
30 *sis thaliana*, *Brassica napus*, *Brassica juncea*, *Musa sp.* (ba-
nana plants), rice, and corn are examples of plant cells
useful in the invention. The skilled person in the art of

plant genetic engineering will know to choose suitable plant
cells in the appropriate stage of their life cycle, suitable
vector systems as well as suitable transformation methods. A
short summary is given here:

5   As other organisms, plant cells can be transformed with
         foreign DNA. One strategy for plant transformation em-
         ploys *Agrobacterium tumefaciens*, a naturally occurring
         plant pathogenic bacterium which contains a plasmid (the
         Ti plasmid) with the ability to enter plant cells and
10   insert a portion of its genome into plant chromosomes.
         The Ti plasmid has been engineered to make it a vector
         for plant transformation by including sequences for re-
         plication in *E. coli* and *Agrobacterium*, unique restric-
         tion sites for inserting foreign genes, and selectable
15   markers.

         Unfortunately, *Agrobacterium* is not very effective at
         transforming monocots, a large group of plants that in-
         cludes all of the agriculturally important cereals. Le-
         gumes, another important group of food crops, are also
20   difficult to transform and regenerate with *Agrobacterium*.
         Therefore, a second strategy for transforming plants has
         been developed involving the Gene Gun, where a gene is
         inserted into an expression vector and coated onto beads.
         The DNA-coated beads are then introduced by means of the
25   gene gun into plant cells, where a small fraction are
         taken up and incorporated into the DNA. Individual plant
         cells, callus, regenerating shoots, and embryos are all
         suitable targets in this technique.

         To determine whether cells have actually incorporated
30   foreign DNA and become transgenic, reporter genes such as
         GUS and Luciferase genes are used. In any case, foreign

genes must be flanked by a plant promoter in order to be
expressed.

It is preferred to use cells derived from animals. These cells
can be mammalian cells, arthropod cells such as insect cells,
5   avian cells, and piscine cells. A number of reasons can be
listed for using such cell types which each require a relevant
choice of transformation and expression systems, growth condi-
tions, etc, all easily determined and chosen by the skilled
person. It suffices to note that e.g. certain insects cause
10 enormous problems in human society (due to their direct damag-
ing activities or due to their functions as vectors carrying
infectious agents), and therefore the method of the invention
would supplement in the attempts of controlling such insects.
As for the mammals, birds and fish, a number of these are
15 important in agri- and aquaculture, where disease control is
of interest.

According to the invention mammalian cells such as human
cells/cell clones or human cell lines are most preferred. This
is due to the fact that a large number of diseases in humans
20 and other mammals etiologically depend on molecular interac-
tions in the living cell - the provision of drugs or lead
compounds which interact *in vivo* with biomolecules which play
a role in diseases is therefore of great interest. Preferred
mammalian cells are Chinese hamster ovary (CHO) cells, VERO
25 cells, HeLa cells, W138 cells, BHK cells, COS-7 293 cells, and
MDCK cells, which are all well-known in the art.

The candidate nucleic acids are hence introduced into eukaryo-
tic cells as part of a vector to screen for modulators of
target enzyme activity. By the term "introduced into" is
30 herein meant that the nucleic acids enter the cells in a
manner suitable for subsequent expression of the nucleic acid.
The method of introduction is largely dictated by the targeted

cell type, cf. below. Exemplary, but non-limiting methods
include CaPO₄ precipitation, liposome fusion, lipofectin®,
electroporation, viral infection, etc.

The randomly modified nucleic acids are preferably integrated
5 into the host cell genome (e.g. by means of retroviral infec-
tion of the host cell), or may exist either transiently or
stably in the cytoplasm (*i.e.* through the use of traditional
plasmids utilizing standard regulatory sequences, selection
markers, etc.).

10 Currently, the most efficient gene transfer methodologies for
mammalian cells harness the capacity of engineered viruses,
such as retroviruses, to bypass natural cellular barriers to
exogenous nucleic acid uptake.

The vector is preferably selected from the group consisting of
15 a retroviral vector, a vaccinia virus vector, an adenoviral
vector, an adeno associated virus (AAV) vector, a herpes
simplex virus (HSV) vector, an alpha virus vector, and a
semliki forest virus vector.

Retroviral transduction

20 As many pharmaceutically important screens require human or
model mammalian cell targets, retroviral vectors capable of
transfecting such targets are preferred.

Therefore, the candidate nucleic acids are preferably part of
a retroviral virion which infects the cells. Generally, infec-
25 tion of the cells is straightforward with the application of
the infection-enhancing reagent polybene. Infection can be
optimized such that the cells predominantly express a single
construct each, e.g. by using the ratio of virus particles to
the number of cells. Alternatively, it is possible to "screen

out" cells which have been infected with more than one single
virion, e.g. by quantitatively assessing a selection marker
and only. The rate of infection is well-known to follow a
Poisson distribution.

5 A preferred embodiment of the invention where the substan-
   tially identical cells are eukaryotic thus comprises that step
   (a) is carried out by
   1)   transfecting suitable packaging cells with vectors com-
         prising the randomly modified nucleotide sequences and
10   which are integratable in virions produced by said
         packaging cells,
   2)   culturing said transfected packaging cells in a culture
         medium under conditions which facilitate production by
         the packaging cells of virions containing the randomly
15   modified nucleotide sequences,
   3)   recovering and optionally concentrating said virions, and
   4)   transducing said substantially identical cells with the
         virions.

Thus, preferably the candidate nucleic acids are introduced
20 into the substantially identical cells using retroviral vec-
    tors. The use is well-known in the art of helper-defective
    packaging cell-lines which are capable of producing all neces-
    sary proteins (*gag*, *pol*, and *env*) required for packaging,
    processing, reverse transcription, and integration of recombi-
25 nant genomes, cf. the below discussion of such cell lines.
    Those RNA molecules which have in *cis* a ψ packaging signal are
    packaged into maturing virions. In eukaryotes, retroviruses
    are preferred for a number of reasons. First, their derivation
    is fairly easy. Second, unlike Adenovirus-mediated gene deli-
30 very, expression from retroviruses is long-term (adenoviruses
    do not integrate). Adeno-associated viruses have limited space
    for genes and regulatory units and there is some controversy
    as to their ability to integrate. Retroviruses therefore

currently provide the best compromise in terms of long-term
expression, genomic flexibility and stable integration, among
other features. The main advantage of retroviruses is that
their integration into the host genome allows for their stable
5   transmission through cell division. This ensures that in cell
types which undergo multiple independent maturation steps,
such as hematopoietic cell progression, the retrovirus con-
struct will remain resident and continue to express.

Preferred retroviral vectors include vectors derived from
10 retrovirus selected from the group consisting of Avian
Leukosis-Sarcoma Virus (ALSV), Mammalian type C, Mammalian
type B, and Lentivirus as well as vectors derived from MSCV
(murine stem cell virus), modified MFG virus and pBABE, and
optionally modified with heterologous cis-acting elements.

15 In general, retroviral vectors should contain as few viral
sequences as possible in order to minimize potential recombi-
nation events. Only the sequences necessary for packaging,
reverse transcription and integration should be retained, as
well as the viral promoter, enhancer, and polyadenylation
20 sequences.

The library of random nucleotides can be generated in a retro-
virus DNA construct backbone, as is generally described in
e.g. WO 97/27212. Standard oligonucleotide synthesis generates
the random portion of the candidate modulator, using tech-
25 niques well known in the art (cf. Eckstein, Oligonucleotides
and Analogues, A Practical Approach, IRL Press At Oxford
University Press, 1991); oligonucleotide libraries may be
commercially purchased. Libraries with up to 10^9 unique sequen-
ces can be readily generated in such DNA backbones. After
30 generation of the DNA library, the library is cloned into a
first primer. The first primer serves as a "cassette" which is
inserted into the retroviral construct. The first primer gene-

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rally contains a number of elements, including for example,
the required regulatory sequences (e.g. translation, tran-
scription, promoters, etc), fusion partners and scaffold
molecule(s), restriction endonuclease (cloning and subcloning)

5   sites, stop codons (preferably in all three reading frames),
regions of complementarity for second strand priming (prefer-
ably at the end of the stop codon region as minor deletions or
insertions may occur in the random region), etc.

A second primer is then added, which generally consists of

10 some or all of the complementarity region to prime the first
primer and optional necessary sequences for a second unique
restriction site for subcloning. DNA polymerase is added to
make double-stranded oligonucleotides. The double-stranded
oligonucleotides are cleaved with the appropriate subcloning

15 restriction endonucleases and subcloned into the target retro-
viral vectors, described below.

In this manner the primers create a library of fragments, each
containing a different random nucleotide sequence within a
scaffold sequence derived from genetic material encoding a

20 enzyme modulator. The ligation products are then transformed
into bacteria, such as *E. coli* and DNA is prepared from the
resulting library, as is generally outlined in Kitamura, PNAS
U.S.A. 92: 9146-50 (1995), which is incorporated by reference
herein.

25 Any number of suitable retroviral vectors may be used. Gene-
rally, the retroviral vectors may include: selectable marker
genes under the control of internal ribosome entry sites
(IRES), which allows for bicistronic operons and thus greatly
facilitates the selection of cells expressing peptides at

30 uniformly high levels; and promoters driving expression of a
second gene, placed in sense or anti-sense relative to the 5'-
LTR (long terminal repeat). Suitable selection genes include,

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but are not limited to, neomycin, blastocidin, bleomycin, puromycin, and hygromycin resistance genes, as well as self-fluorescent markers such as green fluorescent protein, enzymatic markers such as *lacZ*, and surface proteins such as CD8,

5 etc.

The retroviruses may include inducible or constitutive promoters. For example, there are situations wherein it is necessary to induce peptide expression only during certain phases of the selection process. For instance, a scheme to provide

10 pro-inflammatory cytokines in certain instances must include induced expression of the peptides. This is because there is some expectation that over-expressed pro-inflammatory drugs might in the long-term be detrimental to cell growth. Accordingly, in this situation constitutive expression is unde-

15 sirable, and the peptide in only turned on during that phase of the selection process when the phenotype is required, and then the peptide is shut down by turning off the retroviral expression to confirm the effect or ensure long-term survival of the producer cells. A large number of both inducible and

20 constitutive promoters are known to the skilled person.

In addition, it is possible to configure a retroviral vector to allow inducible expression of retroviral inserts after integration of a single vector in target cells; importantly, the entire system is contained within the single retrovirus.

25 Tet-inducible retroviruses have been designed incorporating the Self-Inactivating (SIN) feature of 3'LTR enhancer/promoter retroviral deletion mutant (Hoffmann *et al.*, PNAS U.S.A. 93:5185 (1996)). Expression of this vector in cells is virtually undetectable in the presence of tetracycline or other

30 active analogues. However, in the absence of Tet, expression is turned on to maximum within 48 hours after induction, with uniform increased expression of the whole population of cells that harbour the inducible retrovirus, indicating that expres-

sion is regulated uniformly within the infected cell popula-
tion. A similar, related system uses a mutated Tet DNA-binding
domain such that it bound DNA in the presence of Tet, and was
removed in the absence of Tet. Either of these systems is
5   suitable.

According to the present invention, the most preferred vectors
(described in Example 1) are based on the murine Akv retro-
virus, a mammalian type C retrovirus (NCBI taxonomy Id
#11791). The Akv virus has high homology with the Moloney
10   retrovirus, commonly used in the field. A brief description of
the design of these preferred vectors is as follows:

-     The vectors contain a chimeric 5' LTR, allowing expres-
      sion from the strong Cytomegalovirus (CMV) promoter when
      transcription is driven from the plasmid (as in transfec-
15   tions). Following integration of the vector into the host
      genome, transcription is driven from the retroviral LTR
      (as in transductions).

-     A versatile polylinker is present downstream of the pack-
      aging signal. This enables the insertion of peptide li-
20   braries being part of a scaffold molecule in this posi-
      tion.

-     Immediately downstream of the polylinker is an internal
      ribosomal entry site (IRES), derived from the
      encephalomyocarditis (EMC) virus or an internal promoter,
25   originating from the SV-40 virus. This allows efficient
      translation from the downstream expression cassette ei-
      ther in a CAP independent (IRES), or in a CAP dependent
      (internal promoter) manner.

-     Several different marker genes have been cloned into the
30   downstream expression cassette. For example antibiotics

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resistance genes such as Neo and Hygro, fluorescent pro-
teins such as EGFP, or surface proteins such as ΔNGFR.
The availability of vectors containing different markers
allows for the selection of transduced cells using either

5   drug treatment, flow cytometry or magnetic bead separa-
tion.

–   Any scaffold protein or marker can be combined, either in
the bicistronic vector or in a vector containing the SV-
40 internal promoter.

10 In view of the above, it is hence preferred that the retrovi-
ral vector has non-identical ends so as to facilitate PCR-
based generation of random DNA sequences. It is furthermore
preferred that these non-identical ends contain non-identical
promoters. An especially preferred retroviral vector contains

15 a heterologous promoter replacing the viral promoter in the
5'-LTR, such as a CMV promoter, an RSV promoter, an SV-40
promoter, a TK promoter, an MT promoter, or an inducible
system such as Tet or Ecdysone.

Particularly well suited retroviral transfection systems

20 (packaging cells) are PE501 (US 4,861,719), Bosc23 (WO
94/19478), Ψ2 (R. Mulligan/D. Baltimore), GP+E86 (US
5,278,056), PhoenixEco (WO 97/27212), PA317 (US 4,861,719),
GP+AM12 (US 5,278,056), DA(ampho) (WO 95/10601, WO 92/05266),
Bing (WO 94/19478), FLYA13 (WO 97/08330), ProPak (available

25 from SyStemix), CRIP (R. Mulligan), ΨAM (R. Mulligan/D. Balti-
more), Phoenix-Ampho (WO 97/27212), PG13 (Targeted Genetics),
H9 (293GPG) (D. Ory, M Sadelain, R. Mulligan, J. Schaffer),
and EcoPack (Clonetech).

Retroviral transduction is dependent upon the interaction

30 between the virus envelope glycoproteins and host cell surface
receptors. By far the two most commonly exploited receptors

for retroviral gene delivery are the ecotropic receptor (re-
stricted to murine and rat cells) and the amphotropic receptor
(widely distributed on both immortalized cell lines and on
primary mammalian cells). A number of packaging cell lines,

5 for generating either ecotropic or amphotropic viruses, ex-
ists. In addition, packaging cell lines, which pseudotype
retroviral particles with either GALV (Gibbon Ape Leukemia
Virus glycoprotein) or VSV G (Vesicular Stomatitis Virus G
glycoprotein) have also recently been developed.

10 Until recently most packaging cell lines were based on NIH-3T3
cells, such as the Ψ2 and GP+86 (ecotropic) lines and the
PA317, GP+AM12 and CRIP (amphotropic) lines. These have been
used extensively and work well, particularly when stable
producer lines are made. However, NIH-3T3 based packaging cell

15 lines give relatively low titers when virus is generated from
transient transfection. Over the past few years several pack-
aging cell lines based on the highly transfectable 293 cell
line (human embryonic kidney cells) have been developed. These
include the Bosc23 cell line (Pear *et al.*, 1993) an ecotropic

20 cell line which has been demonstrated to work very well within
the boundaries of the present invention, as well as the Eco-
Pack cell line from Clonetech. The advantage of 293 based
lines is that very high titers (up to 10^7 infectious units/ml,
IU/ml) of viral supernatant can be produced from transient

25 transfections in as little as 48 hours. In a library situation
transient transfections are preferred, as this gives all
library members a chance of being equally well expressed,
without any bias introduced by expression from different
integration sites.

30 Having access to well characterised stable packaging cell
lines is critical for gene therapy associated projects. For
the purposes of the present invention, however, it is not
necessary to employ such well defined lines, as the libraries

will always be produced from transient transfections and no
stable "producer line" will or need be established. An alter-
native strategy for gaining entry into non-murine cells that
has been explored by the present inventors is therefore to use
5   a heterologous viral envelope glycoprotein to pseudotype
viruses produced in ecotropic packaging cell lines, e.g. that
from Vesicular Stomatitis Virus (the VSV G protein). VSV G
pseudotyping of retroviruses is interesting for two main
reasons. First, the cell surface receptors for VSV G are
10 ubiquitous membrane components, such as phosphatidylserine and
gangliosides. Pseudotyping with VSV G therefore confers broad
tropism to the virions. Second, the VSV G protein is extremely
stable once incorporated into the virions. This is important
as it allows concentration of the viral supernatant by ultra-
15 centrifugation, a step which can increase the viral titers per
volume unit by 10 to 100 fold.

The VSV G protein is highly fusogenic and, as a consequence,
exhibits cytotoxicity in tissue culture. It has therefore not
been possible to establish packaging cells that express VSV G
20 constitutively. To circumvent this problem, inducible systems
have been developed (Ory et al., 1996). However, because the
CellScreen™ retroviral libraries are produced from transient
transfections, a very simple alternative is to transiently
transfect VSV G, together with the library, into an ecotropic
25 packaging cell line, such as Bosc23. This approach allows
viral supernatants of broad tropism and high titers to be
produced, before severe toxicity is observed in the culture.
Using this method, a panel of non-murine target cell lines
have been tested for transducability and titers of up to 10^7
30 IU/ ml of viral supernatants have routinely been achieved by
the inventors.

A recently reported alternative to pseudotyping with VSV G is
to pseudotype with the envelope glycoprotein of Lymphocytic

Choriomeningitis Virus (LCMV) (cf. Miletic *et al.*, 1999, J. Virol. 73; 6114-6116). This alternative is also included as an embodiment of the present invention.

After production in packaging cells, concentration of virus
5 may be performed as follows: Generally, retroviruses are titred by applying retrovirus-containing supernatant onto indicator cells, such as NIH-3T3 cells, and then measuring the percentage of cells expressing phenotypic consequences of infection. The concentration of the virus is determined by
10 multiplying the percentage of cells infected by the dilution factor involved, and taking into account the number of target cells available to obtain a relative titre. If the retrovirus contains a reporter gene, such as *lacZ*, then infection, integration, and expression of the recombinant virus is mea-
15 sured by histological staining for *lacZ* expression or by flow cytometry (FACS). In general, retroviral titres generated from even the best of the producer cells do not exceed 10^7 per ml, unless concentration is performed on relatively expensive or exotic apparatus. However, it is believed that particles as
20 large as retrovirus will not move very far by means of brown-ian motion in liquid, fluid dynamics predictions show that much of the virus never comes in contact with the cells in order to initiate infection. However, if cells are grown or placed on a porous filter surface and retrovirus are allowed
25 to pass the cells by gradual gravitometric flow, a high con-centration of virus around cells can be effectively maintained at all times. Thus, up to a ten-fold higher infectivity by infecting cells on a porous membrane and allowing retrovirus supernatant to flow past them has been seen. This should allow
30 titres of 10^9 after concentration.

Upon isolation/concentration of virus, the substantially identical target cells are transduced by methods well-known in the art.

In applications when effector molecules with oncogenic poten-
tial are present in the library it is important to use retro-
viral supernatants which are non-infectious to humans. In
these cases the ecotropic receptor can be stably introduced
5 into the target cell of interest and viruses can be produced
using ecotropic systems. The ecotropic receptor is a cationic
amino acid transporter protein (mCAT), shown to be sufficient
to confer susceptibility to ecotropic virus infection (Albrit-
ton et al. 1989). Expression of this receptor in a variety of
10 human cells, including lymphocytes, have been documented in
the literature (Hitoshi et al., 1998). The present inventors
have demonstrated that introduction of mCAT, both by stable
transfection and by transduction (using a retroviral vector
encoding mCAT), yield target cells that are highly susceptible
15 to infection by Bosc23 generated virions.

Hence, the cell lines discussed above, and the other methods
for producing retrovirus, are useful for production of virus
by transient transfection. The virus can be either used di-
rectly or used to infect another retroviral producer cell so
20 as to expand the library.

Fusion partners

As mentioned above, fusion of the expression product to at
least one fusion partner which facilitates expression and/or
purification/isolation and/or further stabilization of the
25 expression product is often desired.

In eukaryotes, the fusion partner is often a sorting signal or
a targeting sequence. Such a sorting signal will be in the
form of a signal patch or a signal peptide. The well-known
function of a sorting signal is to effect export of an ex-
30 pressed peptide into endoplasmic reticulum, into Golgi appara-
tus, into lysosomes, into secretory vesicles, into mitochon-

dria, into peroxisomes, or into the nucleus. Of course, also
export to the membrane or out of the cell are possibilities.
Preferably, the sorting signal or targeting sequence is se-
lected from the group consisting of

5   -   a nuclear localization signal (NLS) such as Pro-Lys-Lys-
Lys-Arg-Lys-Val (SV40 large T antigen NLS), Ala-Arg-Arg-
Arg-Arg-Pro (human retinoic acid receptor-β NLS), Glu-
Glu-Val-Gln-Arg-Lys-Arg-Gln-Lys-Leu (NFκB p50), Glu-Glu-
Lys-Arg-Lys-Arg-Thr-Tyr-Glu (NFκB p65), and Ala-Val-Lys-

10   Arg-Pro-Ala-Ala-Thr-Lys-Lys-Ala-Gly-Gln-Ala-Lys-Lys-Lys-
Lys-Leu-Asp (Xenopus nucleoplasmin NLS);

  -     a membrane anchoring sequence such as those derived from
CD8, ICAM-2, IL-8, CD4, and LFA-1, and a lipidation se-
quence such as a myristylation or a palmitoylation se-

15   quence;

  -     a lysosomal sorting signal such as a lysosomal degrada-
tion sequence, and a lysosomal membrane sequence

  -     a mitochondrial localization sequence such as a mitochon-
drial matrix sequence, a mitochondrial inner membrane

20   sequence, a mitochondrial intermembrane space sequence,
and a mitochondrial outer membrane sequence; an endoplas-
mic reticulum localization sequence such as the sequence
from calreticulin (KDEL) and the sequence from adenovirus
E3/19K protein (LULSRRSFIDEKKMP);

25   -   a peroxisome sequence such as the peroxisome matrix se-
quence from Luciferase;

  -     a farnesylation sequence such as LNPPDESGPGCMSCKCVLS;

  -     a geranylgeranylation sequence such as LTEPTQPTRNQCCSN;

  -     a destruction sequence such as RTALGDIGN; and

30   -   a secretory signal sequence such as the secretory signals
from IL-2, growth hormone, preproinsulin, and influenza
HA protein.

40

Enzyme modulators useful in the invention

The art has demonstrated the existence of numerous effective
peptide enzyme activity modulators. Especially the enzyme
inhibitors are well-characterized. Non-limiting examples which
5   are all incorporated by reference herein are listed in the
following:

BPTI/Kunitz family of protease inhibitors:
Pancreatic trypsin inhibitor (BPTI) from *Bos taurus;*
Spleen trypsin inhibitor from *Bos taurus;*
10 Inter-alpha-trypsin inhibitor light chain (bikunin) from *Bos*
taurus, Homo sapiens, Meriones unguiculatus, Mesocricetus
auratus, Mus musculus, Sus scrofa, Pleuronectes platessa, and
Rattus norvegicus, respectively;
Inter-alpha-trypsin inhibitor from *Equus caballus, Ovis aries,*
15 and *Capra hircus,* respectively;
Hemolymph trypsin inhibitor A from *Manduca sexta;*
Hemolymph trypsin inhibitor B from *Manduca sexta;*
Colostrum trypsin inhibitor from *Bos taurus;*
Trypstatin from *Rattus norvegicus;*
20 Proteinase inhibitor from *Tachypleus tridentatus;*
Serum basic protease inhibitor from *Bos taurus;*
Chymotrypsin inhibitor SCI-III from *Bombyx mori;*
Male accessory gland serine-protease inhibitor from *Drosophila*
funebris;
25 Protease inhibitor 5 II from *Anemonia sulcata;*
Chymotrypsin inhibitors SCI-I and SCI-II from *Bombyx mori;*
Proteinase inhibitors SHPI and SHPI-2 from *Stoichactis helian-*
thus;
Isoinhibitor K from *Helix pomatia;*
30 Trypsin inhibitor IV from *Radianthus macrodactylus;*
Venom basic protease inhibitors IX and VIIIB from *Bungarus*
fasciatus;

Venom basic protease inhibitors I and III from *Vipera ammo-dytes ammodytes*;

Venom basic protease inhibitor II from *Daboia russelli siamen-sis, Hemachatus haemachatus,* and *Naja nivea,* respectively;

5 Venom basic protease inhibitors B and E from *Dendroaspis polylepis polylepis*;

Venom chymotrypsin inhibitor from *Naja naja*;

Venom basic protease inhibitors I and K from *Dendroaspis polylepis polylepis*;

10 Venom basic protease inhibitor K from *Dendroaspis angusticeps*

Venom trypsin inhibitor from *Eristocophis macmahoni* and *Naja naja,* respectively;

Protease inhibitor from *Sarcophaga bullata*;

Tissue factor pathway inhibitor from *Homo sapiens, Oryctolagus*

15 *cuniculus,* and *Rattus norvegicus,* respectively;

Tissue factor pathway inhibitor 2 from *Homo sapiens*;

Uterine plasmin/trypsin inhibitor from *Sus scrofa*;

Protease nexin II (fragment of Alzheimer's disease amyloid A4 protein) from *Homo sapiens, Mus musculus, rattus norvegicus,*

20 *Macaca fascicularis* and *Macaca mulatta,* respectively;

Amyloid protein 2 from *Homo sapiens* and *Rattus norvegicus,* respectively; and

Ornithodorin from *Ornithodoros moubata,*

as well as inhibitors homologous therewith isolated from other

25 sources than those explicitly mentioned.

Serpin family of protease inhibitors:

Alpha-1-proteinase inhibitors (alpha-1-antitrypsins)from *Equus caballus, Mus musculus, Cavia porcellus, Oryctolagus cunicu-lus, Bos taurus, Chinchilla villidera, Didelphis marsurpiales*

30 *virginiana, Homo sapiens, Macropus eugenii, Mus caroli, Papio anubis, Sus scrofa, Rattus norvegicus,* and *Ovis aries,* respec-tively;

Alpha-1-antichymotrypsin from *Homo sapiens*;

Antithrombin III from *Homo sapiens, Bos taurus, Mus musculus, Ovis aries, Mesocricetus auratus,* and *Gallus gallus,* respectively;

Alpha-2-plasmin inhibitor (alpha-2-antiplasmin) from *Bos*

5   *taurus, Homo sapiens,* and *Mus musculus,* respectively;

Bomapin (Protease Inhibitor 10) from *Homo sapiens;*

Contrapsin from *Mus musculus* and *Cavia porcellus;*

Contrapsin-like protease inhibitors from *Rattus norvegicus;*

Factor XIIa inhibitor from *Bos taurus;*

10 Glia derived nexin (protease nexin I) from *Homo sapiens, Mus musculus,* and *Rattus norvegicus,* respectively;

Heparin co-factor II from *Homo sapiens, Mus musculus, Oryctolagus cuniculus,* and *Rattus norvegicus,* respectively;

47 kDa heat shock protein (serine protease inhibitor J6) from

15 *Mus musculus* and *Gallus gallus,* respectively;

C1-inhibitor from *Homo sapiens;*

Leukocyte elastase inhibitor from *Equus caballus, Homo sapiens,* and *Sus scrofa,* respectively;

Protein C inhibitor from *Homo sapiens;*

20 Kallistatin from *Homo sapiens;*

Kallikrein-binding protein from *Mus musculus* and *Rattus norvegicus,* respectively;

Maspin from *Homo sapiens, Mus musculus,* and *Rattus norvegicus,* respectively;

25 Plasminogen activator inhibitor-1 from *Bos taurus, Homo sapiens, Mus musculus, Mustela vison,* and *Rattus norvegicus,* respectively;

Plasminogen activator inhibitor-2 from *Homo sapiens, Mus musculus,* and *Rattus norvegicus,* respectively;

30 Neuroserpin from *Homo sapiens, Mus musculus,* and *Gallus gallus*

Cytoplasmic antiproteinases 1, 2 and 3 from *Homo sapiens*

Antitrypsin from *Bombyx mori,* respectively;

Antichymotrypsins I and II from *Bombyx mori;*

Alaserpin from *Manduca sexta;*

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Serine protease inhibitor 2.1 from *Rattus norvegicus*;

Serine proteinase inhibitor 1 from Cowpox virus, Rabbit pox virus, Swine pox virus, Vaccinia virus, and Variola virus, respectively;

5 Serine proteinase inhibitor 2 from Rabbit pox virus, Vaccinia virus, and Variola virus, respectively;

Serine proteinase inhibitor 3 from Vaccinia virus and Variola virus, respectively;

Serpin 1 from Myxoma virus;

10 Serine proteinase inhibitor from *Halocynthia roretzi*;

Ice inhibitor from Cowpox virus (thiol-protease inhibitor);

as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

Kazal family of protease inhibitors:

15 Acrosin inhibitors from *Bos taurus, Homo sapiens, Sus scrofa,* and *Macaca fascicularis*, respectively;

Elastase inhibitor from *Anemonia sulcata*;

Ovoinhibitor from *Gallus gallus*;

Rhodniin from *Rhodnius prolixus*;

20 Pancreatic secretory trypsin inhibitor from *Rattus norvegicus, Anguilla anguilla, Bos taurus, Canis familiaris, Homo sapiens, Sus scrofa* and *Ovis aries*, respectively;

Pancreatic secretory trypsin inhibitor II from *Rattus norvegicus*;

25 Double-headed protease inhibitor (submandibular gland) from *Canis familiaris, Felis silvestris catus, Meles meles, Panthera leo,* and *Vulpes vulpes*, respectively;

Trypsin inhibitor from *Halocynthia roretzi*;

Tryptase inhibitor from *Hirudo medicinalis*;

30 Prostatic secretory glycoprotein from *Mus musculus* ;

Ovomucoid (third domain) from *Aburria pipile, Aepypodius arfakianus, Afropavo congensis, Alectoris chukar, Alectoris rufa, Anas platyrhynchos, Chloephaga picta, Cyanochen cyanop-*

tera, Neochen jubata, Tadorna radjah, Lophonetta speculario-
ides, Anas capensis, Aix galericulata, Aix sponsa, Sarkidior-
nis melanotos, Alopochen aegyptiaca, Mergus cucullatus, Anhin-
ga novaehollandiae, Anser anser anser, Anser indicus, Ansera-

5 *nas semipalmata, Arborophila torqueola, Argusianus argus,*
Aythya americana, Netta rufina, Balearica pavonina, Bambusi-
cola thoracica, Bonasa umbellus, Branta canadensis, Anser
canagicus, Callipepla squamata castanogastric, Callipepla
squamata pallida, Carpodacus mexicanus, Carpococcyx renauldi,

10 *Casuarius casuarius, Casuarius bennetti, Cereopsis*
novaehollandiae, Chauna chavaria, Chauna torquata, Gallus
gallus, Chrysolophus amherstiae, Chrysolophus pictus, Circus
aeruginosus, Colinus virginianus, Corvus albus, Corvus monedu-
la, Coscoroba coscoroba, Coturnix delegorguei, Coturnix cotur-

15 *nix japonica, Crossoptilon crossoptilon, Cygnus atratus,*
Cygnus olor, Oxyura jamaicensis, Oxyura vittata, Cyrtonyx
montezumae, Dacelo novaeguineae, Dendrocygna arborea,
Dendrocygna arcuata, Dendrocygna autumnalis, Dendrocygna
bicolor, Dendrocygna eytoni, Dendrocygna viduata, Dromaius

20 *novae-hollandiae, Eudromia elegans, Francolinus afer coqui,*
Francolinus erckelii, Francolinus francolinus, Francolinus
pondicerianus, Fulica atra, Gallinula chloropus, Gallirallus
australis, Gallus varius, Geococcyx californianus, Grus
carunculatus, Grus japonensis, Grus vipio, Anthropoides virgo,

25 *Guira guira, Guttera pucherani, Gyps coprotheres, Polyboroides*
radiatus, Aquila audax, Necrosyrtes monachus, Haliaeetus albi-
cilla, Haliastur indus, Larus ridibundus, Larus marinus,
Vanellus spinosus, Leipoa ocellata, Lophura bulweri, Lophortyx
californica, Lophortyx gambelii, Lophura ignita, Lophura

30 *diardi, Lophura leucomelana, Megapodius freycinet, Meleagris*
gallopavo, Agriocharis ocellata, Nothoprocta cinerascens,
Nothoprocta perdicaria, Numida meleagris, Acryllium vulturi-
num, Nycticorax nycticorax, Opisthocomus hoazin, Oreortyx
pictus, Ortalis vetula, Pavo cristatus, Pavo muticus, Penelope

jacquacu, Penelope superciliaris, Perdrix perdrix, Phasianus colchicus colchicus, Phasianus versicolor, Phalacrocorax sulcirostris, Podargus strigoides, Polyplectron bicalcaratum, Polyplectron emphanum, Polyborus plancus, Pygoscelis adelie,

5 *Phalacrocorax albiventer, Rhea americana, Pterocnemia pennata, Rhynchotus rufescens, Rollulus roulroul, Scythrops novaehollandiae, Spheniscus humboldti, Struthio camelus, Syrmaticus mikado, Syrmaticus reevesii, Tinamus major, Turdus merula, Turnix sylvatica, Tympanuchus cupido, Centrocercus*

10 *urophasianus, Tragopan blythii, Tragopan caboti, Tragopan satyra, Tragopan temminckii, Lophophorus impejanus, Crossopti-lon auritum, Crossoptilon mantchuricum, Lophura edwardsi, Lophura nycthemera, Lophura swinhoei, Pucrasia macrolopha, Catreus wallichii, Syrmaticus ellioti, Syrmaticus humiae,*

15 *Syrmaticus soemmerringii, Lagopus leucurus,* and *Vultur gry-phus,* respectively,
   as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

   Soybean trypsin inhibitor (Kunitz) family of protease inhibi-

20 ***tors:***
   Aspartic proteinase inhibitor from *Solanum tuberosum;*
   Cathepsin D inhibitors from *Solanum tuberosum;*
   Wound-induced aspartate proteinase inhibitor from *Solanum tuberosum;*

25 Chymotrypsin inhibitor 3 precursor from *Psophocarpus tetragonolobus;*
   Trypsin inhibitor from *Adenanthera pavonina;*
   Trypsin inhibitor from *Prosopsis juliflora;*
   Trypsin inhibitor from *Erythrina caffra;*

30 Trypsin inhibitor from *Erythrina latissima;*
   Chymotrypsin inhibitor from *Erythrina variegata;*
   Trypsin inhibitors 1A, 1B, and 2 from *Psophocarpus tetragonolobus;*

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Trypsin/chymotrypsin inhibitor from *Alocasia macrorrhiza*;

Trypsin inhibitor from *Albizzia julibrissin*;

Trypsin inhibitor from *Acacia confusa*;

Trypsin inhibitors A, B and C from *Glycine max*;

5 Trypsin inhibitors KTI1and KTI2 from *Glycine max*;

Latex serine proteinase inhibitor from *Carica papaya*;

Cysteine proteinase inhibitor PCPI 8.3 from *Solanum tuberosum*;
and

Kunitz-type inhibitors 1 and 2 (PKI-1) from *Solanum tuberosum*,

10 as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned

Potato inhibitor I family (protease inhibitors):

Trypsin/subtilisin inhibitor from *Amaranthus caudatus*;

Subtilisin inhibitor from *Momordica charantia*;

15 Wound-induced proteinase inhibitor I from *Lycopersicon
esculentum, Lycopersicon peruvianum*, and *Solanum tuberosum*,
respectively;

Subtilisin inhibitors I and II from *Phaseolus angularis*;

Proteinase inhibitor I from *Solanum tuberosum*;

20 Chymotrypsin inhibitor 2A (CI-2A) from *Hordeum vulgare*;

Chymotrypsin inhibitor 2B (CI-2B) from *Hordeum vulgare*;

Chymotrypsin inhibitor 1A (CI-1A) from *Hordeum vulgare*;

Chymotrypsin inhibitor 1B (CI-1B) from *Hordeum vulgare*;

Chymotrypsin inhibitor 1C (CI-1C) from *Hordeum vulgare*;

25 Chymotrypsin inhibitor I, a, b and c subunits from *Solanum
tuberosum*;

Subtilisin inhibitor from *Vicia faba*;

Ethylene-responsive proteinase inhibitor from *Lycopersicon
esculentum*;

30 Proteinase inhibitors I-A and I-B from *Nicotiana tabacum*;

Eglin C from *Hirudo medicinalis*;

Inhibitor of trypsin and Hageman factor from *Cucurbita maxima*;

Trypsin inhibitor MCI-3 from *Momordica charantia*; and

47

Trypsin inhibitor I from *Nicotiana sylvestris*,

as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

Bowman-Birk *family of protease inhibitors:*

5   Bowman-birk type protease inhibitors from *Arachis hypogaea*, *Coix lachryma-jobi*, *Phaseolus angularis*, *Glycine max*, *Triticum aestivum*, *Arachis hypogaea*, *Phaseolus vulgaris*, *Setaria italica*, *Dolichos axillaris*, *Lonchocarpus capassa*, *Oryza sativa*, *Hordeum vulgare*, *Medicago scutellata*, *Phaseolus aureus*,

10 *Phaseolus lunatus*, *Vicia angustifolia*, *Vicia faba*, and *Vigna unguiculata*, respectively; and

Wound induced trypsin inhibitors from *Medicago sativa* and *Zea mays*, respectively,

as well as inhibitors homologous therewith isolated from other

15 sources than those explicitly mentioned.

Squash inhibitor *family:*

Elastase inhibitor from *Momordica charantia*;

Trypsin inhibitors I, II, and III from *Lagenaria leucantha*;

Trypsin inhibitor I from *Citrullus vulgaris*;

20 Trypsin inhibitors I, III, and IV from *Cucurbita maxima*;

Trypsin inhibitors I and II from *Luffa cylindrica*;

Trypsin inhibitors I and II from *Momordica charantia*;

Trypsin inhibitor I from *Momordica repens*;

Trypsin inhibitor II from *Bryonia dioica*;

25 Trypsin inhibitors IIB and IV from *Cucumis sativus*;

Trypsin inhibitor II from *Ecballium elaterium*;

Trypsin inhibitors I, II and III from *Cucumis melo* var. *Conomon*;

Trypsin inhibitors II and III from *Cucurbita pepo*; and

30 Trypsin inhibitor A from *Momordica charantia*,

as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

Streptomyces subtilisin inhibitor (SSI) family:

Subtilisin inhibitor from *Streptomyces albogriseolus*;

Subtilisin inhibitor-like protein-1 from *Streptomyces cacaoi*;

Trypsin inhibitor STI2 from *Streptomyces longisporus*;

5 Subtilisin inhibitor-like protein-2 from *Streptomyces rochei*;

Subtilisin inhibitor-like protein-3 from *Streptomyces coeli-
color*;

Subtilisin inhibitor-like protein-4 from *Streptomyces laven-
dulae*;

10 Subtilisin inhibitor-like protein-8 from *Streptomyces virgin-
iae*;

Protease inhibitor SIL-V3 from *Streptoverticillium eurocidi-
cus*;

Protease inhibitor SIL-V1/SIL-V4 from *Streptoverticillium*

15 *flavopersicus*;

Protease inhibitor SIL-V5 from *Streptoverticillium luteoverti-
cillatus*;

Protease inhibitor SIL-V2 from *Streptoverticillium orinoci*;

Alkaline protease inhibitor 2C' from *Streptomyces griseoincar-

20 natus*;

Protease inhibitor from *Streptomyces lividans*; and

Plasminostreptin from *Streptomyces antifibrinolyticus*,

as well as inhibitors homologous therewith isolated from other

sources than those explicitly mentioned.

25 ***Bombyx family of protease inhibitors***

Fungal protease inhibitor F (FPI-F) from *Bombyx mori*, as well

as inhibitors homologous therewith isolated from other sources

than *Bombyx mori*.

Wap-type 'Four-disulfide Core' Proteinase inhibitors

Antileukoproteinase 1 from *Homo sapiens* and *Mus musculus*, respectively;

Elafin from *Homo sapiens* and *Sus scrofa*, respectively; and

5 Chelonianin (basic protease inhibitor) from red sea turtle, as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

Hirudin family of protease inhibitors

Hirudins from *Hirudo medicinalis* and *Hirudinaria manillensis*,

10 respectively, as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

Factor Xa inhibitors

Antistasin from *Haementeria officinalis, Haementeria ghili-anii, Hirudo medicinalis, Hirudo nipponia*, and *Hydra*

15 *magnipapillata*, respectively, as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

Ascaris trypsin inhibitor family

Chymotrypsin/elastase inhibitors from *Ascaris suum* and

20 Trypsin inhibitor from *Ascaris suum* as well as inhibitors homologous therewith isolated from other sources than *Ascaris suum*.

Cystatin family of protease inhibitors

Leukocyte cysteine proteinase inhibitors 1 and 2 from *Sus*

25 *scrofa;*

Stefins 1, 2 and 3 from *Mus musculus;*

Cystatins A1, A5, A8 and B from *Sus scrofa;*

Cystatins A and B from *Bos taurus;*

Stefin C from *Bos taurus;*

30 Cystatin B from *Ovis aries;*

50

Cystatins A, C, D, M, SN, S and SA from *Homo sapiens;*

Cystatins B and C from *Mus musculus;*

Cystatins C and S from *Rattus norvegicus;*

Cystatins C from *Macaca mulatta* and *Saimiri sciureus,* respec-

5 tively;

Cystatin alpha (epidermal thiol proteinase inhibitor) from

Rattus norvegicus;

Cystatin B (liver thiol proteinase inhibitor) from *Homo sapi-*

ens and *Rattus norvegicus,* respectively;

10 Cystatin (colostrum thiol proteinase inhibitor) from *Bos*

taurus;

Cystatins from *Gallus gallus* and *Coturnix coturnix japonica,*

respectively;

Cystatin from *Cyprinus carpio;*

15 Cystatin from *Bitis arientas;*

Cystatin I from *Zea mays;*

Oryzacystatin-I from *Oryza sativa;*

Oryzacystatin-II from *Oryza sativa;*

Cystatin A from *Helianthus annuus;*

20 Cystatin B from *Helianthus annuus;*

Cystatin from *Vigna unguiculata;*

Onchocystatin from *Onchocerca volvulus;*

Cysteine proteinase inhibitor from *Solanum tuberosum;*

Cystatin WCPI-3 from *Wisteria floribunda;*

25 Cystatin from *Glycine max;* and

Kininogens from *Bos taurus, Homo sapiens* and *Rattus norvegi-*

cus, respectively,

as well as inhibitors homologous therewith isolated from other

sources than those explicitly mentioned.

30 ***Calpain family of cysteine protease inhibitors***

Calpastatin from *Bos taurus, Cercopithecus aethiops, Homo*

sapiens, Mus musculus, Sus scrofa, Oryctolagus cuniculus,

Rattus norvegicus, and *Ovis aries,* respectively,

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as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

Tissue inhibitor of metalloproteinases family

Metalloproteinase inhibitor 1 from *Bos taurus, Homo sapiens,*
5 *Mus musculus, Papio cynocephalus, Sus scrofa, Oryctolagus
cuniculus, Rattus norvegicus,* and *Ovis aries,* respectively;
Metalloproteinase inhibitor 2 from *Bos taurus, Homo sapiens,
Mus musculus, Rattus norvegicus,* and *Gallus gallus,* respec-
tively; and
10 Metalloproteinase inhibitor 3 from *Bos taurus, Homo sapiens,
Mus musculus, Rattus norvegicus,* and *Gallus gallus,* respec-
tively,
as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

15 **Carboxypeptidase A inhibitors**

Carboxypeptidase A inhibitor from *Ascaris suum,* as well as
inhibitors homologous therewith isolated from other sources
than *Ascaris suum.*

Metallocarboxypeptidase inhibitors

20 Metallocarboxypeptidase inhibitors from *Lycopersicon esculen-
tum* and *Solanum tuberosum,* respectively, as well as inhibitors
homologous therewith isolated from other sources than those
explicitly mentioned.

Angiotensin-converting enzyme inhibitors

25 Angiotensin-converting enzyme inhibitor from *Thunnus albaca-
res;*
Angiotensin-converting enzyme inhibitors from *Bothrops insula-
ris;*
Angiotensin-converting enzyme inhibitors from *Bothrops jarara-
30 ca;*

52

Angiotensin-converting enzyme inhibitor from *Agkistrodon halys blomhoffi*;

Angiotensin-converting enzyme inhibitor from *Agkistrodon halys pallas*; and

5   Angiotensin-converting enzyme inhibitor from *Vipera aspis*,
    as well as inhibitors homologous therewith isolated from other
    sources than those explicitly mentioned.

Protease inhibitors not belonging to a specific family

Ecotin from *Escherichia coli*;

10 Metalloproteinase inhibitor from *Streptomyces nigrescens*;
    Proteinase inhibitor from *Erwinia chrysanthemi*;
    Proteinase inhibitor from *Pseudomonas aeruginosa*;
    Protease A inhibitor 3 from *Saccharomyces cerevisiae*;
    Protease B inhibitors 1 and 2 from *Saccharomyces cerevisiae*;

15 Major pepsin inhibitor PI-3 from *Ascaris suum*;
    Intracellular proteinase inhibitor from *Bacillus subtilis*;
    Proteinase inhibitor PTI from *Solanum tuberosum*;
    Proteinase inhibitor PCI-I from *Solanum tuberosum*;
    Proteinase inhibitor IIA from *Solanum tuberosum*;

20 Proteinase inhibitor IIB from *Solanum tuberosum*;
    Proteinase inhibitors A and B from *Sagittaria sagittifolia*;
    Proteinase inhibitor from *Solanum melongena*;
    Trypsin inhibitor from *Brassica napus*;
    Trypsin inhibitor 2 from *Sinapis alba*;

25 Trypsin inhibitor from *Zea mays*;
    Trypsin inhibitor from *Sinapis arvensis*;
    Trypsin inhibitor from *Trichosanthes kirilowii*;
    Wound-induced proteinase inhibitor II from *Lycopersicon esculentum*;

30 Protease inhibitors LCMI I and PMP-D2 from *Locusta migratoria*;
    Protease inhibitor from *Bacillus brevis*;
    Marinostatins C-1, C-2 and D from *Alteromonas* sp.; and
    Host protease inhibitor from bacteriophage T4,

as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

Cereal alpha-amylase/trypsin inhibitor family
Alpha amylase/trypsin inhibitor CM1 from *Triticum aestivum*;
5 Alpha amylase/trypsin inhibitor CM2 from *Triticum aestivum*;
Alpha amylase/trypsin inhibitor CM3 from *Triticum aestivum*;
Alpha amylase/trypsin inhibitor CM16 from *Triticum aestivum*;
Alpha amylase/trypsin inhibitor CM17 from *Triticum aestivum*;
Alpha amylase inhibitor 0.19 from *Triticum aestivum*;
10 Alpha amylase inhibitor 0.28 from *Triticum aestivum*;
Alpha amylase inhibitor 0.53 from *Triticum aestivum*;
Alpha amylase inhibitor WDAI-3 from *Triticum aestivum*;
Alpha amylase/trypsin inhibitor CMA from *Hordeum vulgare*;
Alpha amylase/trypsin inhibitor CMB from *Hordeum vulgare*;
15 Alpha amylase/trypsin inhibitor CMC from *Hordeum vulgare*;
Alpha amylase/trypsin inhibitor CMD from *Hordeum vulgare*;
Alpha amylase inhibitor CME from *Hordeum vulgare*;
Alpha amylase inhibitor BMAI-1 from *Hordeum vulgare*;
Alpha amylase inhibitor BDAI-1 from *Hordeum vulgare*;
20 Alpha amylase/trypsin inhibitor from *Eleusine coracana*; and
Trypsin/factor XIIa inhibitor from *Zea mays*,
as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

Alpha-amylase/trypsin inhibitor homologous to thaumatin
25 Alpha-amylase/trypsin inhibitor from *Zea mays* as well as
inhibitors homologous therewith isolated from other sources
than *Zea mays*.

Alpha-amylase/subtilisin inhibitor family
Alpha-amylase/subtilisin inhibitor from *Hordeum vulgare*;
30 Alpha-amylase/subtilisin inhibitor from *Triticum aestivum*; and
Alpha-amylase/subtilisin inhibitor from *Oryzae sative*,

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as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

Inhibitors of insect alpha-amylases

Small protein inhibitor of insect alpha-amylases 1, 2 and 3
5 from *Sorghum bicolor milo* as well as inhibitors homologous
therewith isolated from other sources than *Sorghum bicolor
milo*.

Inhibitors of mammalian alpha-amylases derived from Streptomyces species

10 Alpha-amylase inhibitor HAIM I from *Streptomyces griseospo-
reus;*
Alpha-amylase inhibitor PAIM I from *Streptomyces olivaceoviri-
dis;*
Alpha-amylase inhibitor HAIM II from *Streptomyces griseospo-
15 reus;*
Alpha-amylase inhibitor PAIM II from *Streptomyces
olivaceoviridis;*
Alpha-amylase inhibitor AI-3688 from *Streptomyces aureofaci-
ens;*
20 Alpha-amylase inhibitor Z-2685 from *Streptomyces rochei;* and
Alpha-amylase inhibitor HOE-467A from *Streptomyces tendae,*
as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

Trehalase inhibitors

25 Trehalase inhibitor from *Periplaneta americana* as well as
inhibitors homologous therewith isolated from other sources.

Polygalacturonase inhibitors

Polygalacturonase inhibitors from *Phaseolus vulgaris* and *Pyrus
communis* as well as inhibitors homologous therewith isolated
30 from other sources than those explicitly mentioned.

Fucosyltransferase inhibitors

Fuctinins from *Rattus norvegicus* as well as inhibitors homologous therewith isolated from other sources.

Protein kinase C inhibitors

5 14-3-3 Protein beta/alpha from *Bos taurus, Ovis aries, Homo sapiens,* and *Rattus norvegicus*, respectively;

14-3-3 Protein epsilon from *Mus musculus, Ovis aries, Homo sapiens,* and *Rattus norvegicus*, respectively;

14-3-3 Protein eta from *Bos taurus, Mus musculus,* and *Rattus*
10 *norvegicus*, respectively;

14-3-3 Protein gamma from *Bos taurus, Ovis aries,* and *Rattus norvegicus*, respectively;

14-3-3 Protein zeta/delta from *Bos taurus, Mus musculus, Ovis aries, Homo sapiens,* and *Rattus norvegicus*, respectively;

15 Hint protein from *Bos taurus, Rattus norvegicus, Homo sapiens, Mus musculus,* and *Oryctolagus cuniculus*, respectively; and

14 kDa zinc-binding protein from *Brassica juncea* and *Zea mays*, respectively,

as well as inhibitors homologous therewith isolated from other
20 sources than those explicitly mentioned.

cAMP-dependent protein kinase inhibitors

cAMP-dependent protein kinase inhibitor (muscle/brain form) from *Homo sapiens, Oryctolagus cuniculus, Mus musculus,* and *Rattus norvegicus*, respectively, and

25 cAMP-dependent protein kinase inhibitor (testis form) from *Mus musculus,* and *Rattus norvegicus*, respectively,

as well as inhibitors homologous therewith isolated from other sources than those explicitly mentioned.

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Cyclic nucleotide phosphodiesterase inhibitor
Cyclic nucleotide phosphodiesterase inhibitor from
Dictyostelium discoideum as well as inhibitors homologous
therewith isolated from other sources.

5 **Protein phosphatase inhibitors**
Protein phosphatase inhibitor 1 from *Homo sapiens, Oryctolagus
cuniculus,* and *Rattus norvegicus,* respectively;
Protein phosphatase inhibitor 2 from *Homo sapiens, Oryctolagus
cuniculus,* and *Rattus norvegicus,* respectively;

10 Heat-stable protein phosphatase 2a inhibitor I1PP2A from *Bos
taurus* and *Homo sapiens,* respectively; and
Phosphatase RAPA inhibitor from *Bacillus subtilis,*
as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

15 **TCD/MRS6 family of GDP dissociation inhibitors**
Secretory pathway GDP dissociation inhibitor from *Saccharomy-
ces cerevisiae;*
Rab GDP dissociation inhibitor alpha from *Bos taurus, Homo
sapiens, Mus musculus,* and *Rattus norvegicus,* respectively;

20 Rab GDP dissociation inhibitor beta from *Homo sapiens, Mus
musculus,* and *Rattus norvegicus,* respectively;
Rho GDP-dissociation inhibitor 1 from *Bos taurus, Homo sapi-
ens, Caenorhabditis elegans* and *Cavia porcellus,* respectively;
Rho GDP-dissociation inhibitor from *Saccharomyces cerevisiae ;*

25 Rho GDP-dissociation inhibitor 2 from *Homo sapiens* and *Mus
musculus,* respectively; and
Rho GDP-dissociation inhibitor from *Mus musculus,*
as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

ATPase inhibitors

Mitochondrial ATPase inhibitors from *Bos taurus, Caenorhab-
ditis elegans, Pichia jadinii, Sus scrofa, Rattus norvegicus,*
and *Saccharomyces cerevisiae,* respectively, and

5 Sodium/potassium ATPase inhibitor from *Sus scrofa,*
as well as inhibitors homologous therewith isolated from other
sources than those explicitly mentioned.

Phospholipase A2 inhibitory proteins

Annexin I from *Bos taurus, Homo sapiens, Mus musculus, Sus*

10 *scrofa, Rattus norvegicus, Oryctolagus cuniculus, Cavia cut-
leri, Gallus gallus,* and *Columba livia,* respectively;
Annexin III from *Homo sapiens* and *Rattus norvegicus,* respec-
tively;
Annexin V from *Homo sapiens, Mus musculus, Rattus norvegicus,*

15 and *Gallus gallus,* respectively;
Uteroglobulin from *Oryctolagus cuniculus* and *Lepus capensis,*
respectively; and
Phospholipase A2 inhibitor from *Trimeresurus flavoviridis,*
as well as inhibitors homologous therewith isolated from other

20 sources than those explicitly mentioned.

Ribonuclease inhibitors

Barstar from *Bacillus amyloliquefaciens;*
Ribonuclease inhibitor from *Homo sapiens, Sus scrofa,* and
Rattus norvegicus, respectively, as well as inhibitors homolo-

25 gous therewith isolated from other sources than those
explicitly mentioned.

RNA polymerase inhibitors

Bacterial RNA polymerase inhibitors from Bacteriophages T3 and
T7 as well as inhibitors homologous therewith isolated from

30 other sources.

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DNA-entry nuclease inhibitors

Competence protein J from *Bacillus subtilis* as well as inhibitors homologous therewith isolated from other sources.

Beta-lactamase inhibitors

5   Beta-lactamase inhibitor from *Streptomyces clavuligerus* as well as inhibitors homologous therewith isolated from other sources.

To this list of enzyme inhibitors can be added functionally related inhibitors such as the inhibitor of calcium transport

10   seminalplasmin from *Bos taurus* and *Mus musculus*, respectively, as well as inhibitors homologous therewith isolated from other sources.

To the above list of suitable scaffold molecules to be used in the invention should be added other agents having an effect on

15   the activity of enzymes. One interesting candidate is thioredoxin.

All of the above-listed scaffold molecules can be substituted with an effective part of the complete molecule.

Properties of the scaffold molecule

20   Since the peptide library presumably can reach every compartment of a cell, it is beneficial if the scaffold enzyme inhibitor protein is not too large, and that it is stable towards e.g. proteolytic attack and insensitive to the reducing environment inside eukaryotic cells. Hence, its function

25   should preferably not be dependent on the formation of disulfide bridges, since these are not formed in the cytosol or nucleus of such cells. In addition the scaffold protein should contain one or more exposed loops in which peptides can be inserted without markedly changing the structure or stability

of the protein. On this basis the chymotrypsin inhibitor 2A
(cf. the discussion below) is a suitable scaffold but other
scaffolds such as the examples mentioned below could also be
used.

5 The members of the peptide library introduced into target
cells can effect change of the target cells' phenotype either
by direct or indirect (transdominant) interaction. However,
the method of the invention renders possible the identifica-
tion of not only the peptide or ribonucleic acid sequence
10 which is responsible for the direct or indirect effect on
phenotype, but also allows for the purification, isolation and
identification of the target molecule with which the peptide
interacts.

In the present specification the barley-derived chymotrypsin
15 inhibitor 2A has been used by way of example, mainly because
this protein is very well-characterized and extremely stable.
It will be understood, however, that the use of this enzyme
inhibitor in the Examples in no way should limit the scope of
the present invention.

20 Barley chymotrypsin inhibitor 2A belongs to a large family of
homologous protease inhibitors mainly found in plants. This
family includes barley chymotrypsin inhibitors 1A, 1B, 2A and
2B, potato inhibitor I , wound-induced tomato inhibitor I,
ethylene-responsive tomato inhibitor, wild tomato fruit
25 inhibitor I, a subtilisin inhibitor from broad bean, adzuki
bean subtilisin inhibitor, pumpkin trypsin/Hageman factor
inhibitor, bitter gourd inhibitor, protoplast-specific trypsin
inhibitor from Nicotiana sylvestris, tobacco subtilisin inhi-
bitor, amaranth trypsin/subtilisin inhibitor, and beach
30 canavalia subtilisin inhibitor. The only member of this
inhibitor family that is of non-plant origin is the leech
elastase/cathepsin G inhibitor eglin C.

CI-2A was originally purified from the endosperm of the high-lysine barley Hiproly and shown to be a tight binding inhibitor of the microbial subtilisins Carlsberg and Novo as well as of chymotrypsin. The N-terminal amino acid residue in

5 CI-2A has a blocked amino group as direct amino acid sequencing was unsuccessful. However, most of the amino acid sequence of CI-2A has been determined at the protein level. In addition, it was shown that CI-2A purified from barley is N-terminally processed either during synthesis and storage in

10 the endosperm or in the process of purification. The absence of the 17 N-terminal amino acid residues does not influence the complex formation of CI-2A with subtilisins. Combining the results of amino acid sequencing and cDNA sequencing it has been deduced that CI-2A consists of 83 amino acid residues in

15 a single polypeptide chain containing no disulfide bonds. The blocked N-terminal amino acid residue is serine. The reactive site in CI-2A has been determined to be the Met59-Glu60 peptide bond and the residues in the region Ile56-Arg62 have been demonstrated to be involved in the intermolecular contacts

20 between inhibitor and protease.

The three-dimensional structures of uncomplexed CI-2A as well as of CI-2A complexed with subtilisin Novo are known from X-ray crystallography. The three-dimensional structure of CI-2A has also been determined using NMR spectroscopy, reveal-

25 ing that the reactive loop of CI-2A is dynamic. CI-2A consists of a single α-helix docking against four β-strands. The surface loop stretches across the free side of the sheet and is composed of eight residues: Gly54-Tyr61. In contrast to most enzyme inhibitors, CI-2A lacks disulfide bonds as well as

30 glycosylation sites. In the structures determined, only the 64 C-terminal amino acid residues are defined (L20-G83); this truncated version retains the functionality of the native protein. Comparing the complexed form with the two uncomplexed forms of CI-2A reveals few differences. The most notable

difference is that the reactive site loop seems to have a less
ordered structure in the uncomplexed forms than in the
complexed form. The three-dimensional structure of CI-2A in
complex with subtilisin Novo has also been compared to the
5 three-dimensional structure of eglin C in complex with
subtilisin Carlsberg. The two homologous inhibitors have
highly similar secondary and tertiary structures.

Recombinant variants of N-terminally truncated CI-2A
(CI-2A(L20-G83)) and CI-2A with an N-terminal Asp-Pro exten-
10 sion have been widely used to study the folding and stability
of CI-2A. The structure of CI-2A in complex with subtilisin
Novo has revealed that the number of intermolecular contacts
between inhibitor and protease in the P4-P1 region
(Ile56-Met59) of the inhibitor are much larger than in the
15 P1'-P3' region (Glu60-Arg62).

Further aspects of the invention

Having identified a modulator according to the above-detailed
methods of the invention it is normally of interest to provide
large quantities thereof for the purposes of further research
20 and development, including possible identification of the
target molecule with which the modulator physically interacts.

Therefore, the invention also pertains to a method for the
preparation of a replicable expression vector, the method com-
prising the steps of identifying a modulator by the methods of
25 the invention, and subsequently
    -   isolating or synthesizing a nucleic acid sequence which
        encodes the modulator, and
    -   engineering a replicable expression vector comprising an
        operon which comprises, in the 5'-3' direction and in
30   operable linkage, 1) a promoter for driving expression of
        the nucleic acid sequence, 2) optionally a nucleotide

sequence encoding a leader peptide, 3) the nucleic acid
sequence, and 4) a termination signal.

Such methods are widely known in the field of genetic
engineering and molecular biology. The skilled person will
5 find suitable guidance in e.g. Sambrook J, Fritsch EF, Mani-
atis T. 1989. "Molecular cloning: A laboratory manual", 2nd
ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

In this part of the invention it is preferred that the pro-
moter is inducible; constitutive promoters are not excluded
10 though. Depending on the choice of host cell to carry and
express the vector, the promoter is selected from the group
consisting of a bacterial promoter, a fungal promoter such as
a yeast promoter, and a mammalian promoter. The vector can be
in the form of a plasmid, a phage, a cosmid, a minichromosome,
15 or a virus, again depending on choice of host cell and other
considerations applying for the specific case. However, it is
most preferred that the expression vector is capable of being
integrated into the genome of a suitable host cell, since the
expression therein will then be more stable over time than is
20 the case with non-chromosomal transformation of the host cell.

Well-known vector systems are based on bacterial plasmid
pBR322, λ-phage, and yeast plasmid YRp7, but other suitable
and feasible choices are known to the skilled person.

After having provided a suitable expression vector as outlined
25 above, it is preferred that the modulator is produced by
transforming a suitable host cell with an expression vector
prepared as described above. Such a host cell can be bacterial
(e.g. *E. coli*, *Bacillus subtilis*, or any other suitable bacte-
rial host cell), a fungus (e.g. a yeast cell such as
30 *Saccharomyces cerevisiae* or Piccia pastoris) or a plant,
insect or mammalian cell (which can be any of the above-dis-

cussed cells or cell-types suitable for use as the "substan-
tially identical cells" in the method of the invention).

After having provided a producer cell line as described above
it is now possible to produce the modulator of the invention
5 by growing the transformed cell prepared as described above in
a culture medium under conditions which facilitates expression
by the cell of the randomly modified nucleotide sequence, and
subsequently harvesting the expression product from the cell
and/or the culture medium. Alternatively, the modulator can be
10 produced by synthesizing the modulator by means of chemical
synthesis on the basis of the sequence determined in step (e).
In the case that the modulator is a peptide, the well-known
techniques of solid- or liquid-phase peptide synthesis can be
employed and also if the modulator is a ribonucleic acid, me-
15 thods for synthetic production thereof are readily available.

An important part of the invention pertains to the isola-
tion/identification of the target biomolecules which are enga-
ging the modulator, the identification, isolation and produc-
tion of which is described above. Hence an important part of
20 the invention pertains to a method for isolating and/or iden-
tifying a target biomolecule, the method comprising providing
a modulator according to the methods described herein and
subsequently using the modulator as an affinity ligand in an
affinity purification step so as to isolate the target
25 biomolecule from a suitable sample. The affinity purification
step can e.g. be an affinity chromatographic step, an affinity
mass spectrometry step, or a co-immunoprecipitation step.
However, any suitable method for affinity-based isola-
tion/purification can be employed.

30 Alternatively, the modulator can be used as a probe against a
cDNA library derived from the substantially identical cells or

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as bait in a two- or three-hybrid system (bacterial, fungal or mammalian).

It is preferred that the target biomolecule is a peptide or a nucleic acid, since this also allows for sequence determina-
5 tion thereof.

The potential use of such a target biomolecule is to employ it in a drug-development program. For this purpose it is often useful to resolve or get information about the 3-dimensional structure of the target biomolecule (by means of methods
10 available, e.g. X-ray diffraction studies, NMR analysis, circular dichroism, etc).

Having isolated a target biomolecule as described above, the invention allows for the rational selection of a chemical com-pound to be used as a putative drug candidate in drug develop-
15 ment, the method comprising the steps of

-       assaying a library of chemical compounds for interaction
        with a target biomolecule which has been *de novo* isolated
        according to the methods of the invention, and
-       selecting compounds which interact significantly with the
20   target biomolecule.

Such an identified drug candidate can be a lead compound or a drug candidate as such. A "lead compound" is in the present application understood as being a compound which is not in itself suitable as a drug but which exhibits a number of
25 characteristics which are interesting when viewed from the point of view of medical therapy. The reason such a lead compound is unsuitable could be toxicity, unsuitable pharmacokinetic or pharmacodynamic properties, difficulties relating to preparation and purification etc. In such cases,
30 the lead compound is used as a model for *de novo* synthesis of

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other chemical compounds which are designed so as to be re-
lated to the active part of the lead compound in 3D structure
and distribution of charged, polar and non-polar groups.

This approach can be refined by initially identifying the mem-
5 bers of the library by methods of structure-based or non-
structure based computer drug-modelling. Suitable non-struc-
ture based methods are disclosed in US 5,307,287 and US
5,025,388; a method known as CoMFA). An alternative is HASL
(Hypothetical Active Site Lattice; Hypothesis Software). Both
10 these methods are based on 3D-QSAR. A feasible structure-based
approach is e.g. disclosed in WO 95/06293.

Finally, a very important part of the present invention per-
tains to a method for the preparation of a medicinal product,
the method comprising the steps of
15 a)   selecting a chemical compound by the methods of the in-
       vention described above,
   b)   performing pre-clinical tests with the chemical compound
       in order to assess the suitability thereof as a medicinal
       product,
20 c)   entering, if the chemical compound is deemed suitable in
       step (b), clinical trials using the chemical compound in
       order to obtain market authorization for a medicinal
       product including the chemical compound as a pharmaceuti-
       cally active substance, and
25 d)   upon grant of a market authorization, admixing the chemi-
       cal compound with a pharmaceutically acceptable carrier,
       excipient or diluent and marketing the thus obtained
       medicinal product.

In other words, also encompassed by the present invention is a
30 method for developing a medicinal product, the method compri-
sing that a modulator identified according to the method of
the invention serves as a lead compound in the development

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phase or wherein a target biomolecule isolated/identified
according the invention serves as an interaction probe against
putative drug candidates in the drug discovery phase.

The above-outlined methods should of course take into conside-
5 ration all necessary requirements in order to meet GCP and GMP
standards. At any rate, this method is completely depending on
the initial provision of the modulator identified according to
the invention.

LEGEND TO THE FIGURES

10 Fig. 1: A schematic representation of pCMVbipep/CI-2A with the
functional cis-elements found in pCMVbipep indicated.
At the top, a schematic representation of pCMVbipep is pre-
sented and the CI-2A cDNA region is expanded in the illustra-
tions below showing the position of the various signal pep-
15 tides (SEQ ID NOs: 35-37) present in pCMVbipepER/CI-2A,
pCMVbipepNLS/CI-2A, and pCMVbipepSL/CI-2A, respectively. The
extra amino acids added to CI-2A are written in one letter
code with the essential amino acid sequences required for
function underlined. Abbreviations: nuclear localization
20 signal, NLS; endoplasmic reticulum, ER; secretoric leader, SL;
retention signal, RS.

Fig. 2: Total extracts from CMVbipep/CI-2A transduced cells
are capable of inhibiting the protease activity of subtilisin.
Subtilisin was incubated with increasing amounts of extracts
25 from the indicated cell lines either transduced with CMVbipep
(NIH-3T3, U2OSmCAT, and 293mCAT) or CMVbipep/CI-2A (NIH-
3T3/CI-2A, U2OSmCat/CI-2A, and 293mCat/CI-2A) and subsequently
assayed for residual proteolytic activity. Each reaction with
a given extract concentration was determined in triplicate and
30 the shown velocities are based on the mean values.

Fig. 3: Nuclear extract from CMVbipepNLS/CI-2A, but not from
CMVbipep/CI-2A transduced NIH-3T3 cells exerts CI-2A activity.
A) Subtilisin was incubated with either 2 or 10 µl of nuclear
extract from NIH-3T3 cells transduced with either CMVbipep

5 (-), CMVbipep/CI-2A (CI-2A), or CMVbipepNLS/CI-2A (NLS/CI-2A)
and subsequently assayed for proteolytic activity.
B) As for A except that 0.4 or 2 µl total cell extract was
used. Each reaction with a given extract concentration was
determined in triplicate and the shown velocities are based on

10 the mean values.

Fig. 4: Schematic presentation of pFAB60 constructs.
Upper panel: pFAB60/CI-20 contains the truncated version of
CI-2A is inserted in frame with a pelB leader sequence at the
5' end and the deleted gene encoding a fragment of the M13

15 phage surface protein III (ΔgIII) at the 3'end. The pelB
leader directs the expressed fusion protein to the bacterial
membrane thereby facilitating incorporation of the CI-2A/ΔpIII
fusion protein into phage particles.
Middle panel: pFAB60/muCI-2A contains the CI-2A cDNA including

20 the recognition sites for MunII and SalI restriction enzymes.
Lower panel: pFAB60/muCI-2A_rc has amino acid 58-61 substi-
tuted for the underlined 19 randomly composed amino acids (SEQ
ID NO: 38, residues 2-20). The amino acids are shown in one
letter codes and the numbers refers to the unmodified CI-2A

25 amino acid sequence (cf. The numbering in SEQ ID NO: 2).

Fig. 5: CI-2A and CI-2A_rc can be displayed on the surface of
phage particles.
Anti CI-2A antibodies were immobilized and incubated with 0 or
5 x 10^{11} phage particles, produced and purified as in Johansen

30 et al., 1995, Protein Eng. 10, pp. 1063-1067 and quantified by
OD269 measurement. CI-2A negative (-) CI-2A or CI-2A_rc carry-
ing phage particles were used. The retained phage particles
were finally detected by a horse radish peroxidase conjugated

anti phage antibody. The results shown are mean values from 4
independent measurements with indicated standard deviation.

Fig. 6: Construction of a CI-2A presented library using the
*Mun*I and *Sal*I cloning sites.
5 A) A degenerated oligo (SEQ ID NO: 33, residues 21-46) cover-
ing the recognition sites for *Mun*I and *Sal*I is converted to a
double stranded form in a single extension reaction.
B) Schematic presentation of the modified cDNA of a CI-2A
peptide library.
10 C) Three-dimensional structure of the 64 C-terminal amino
acids. The substituted amino acids are shown in white with the
sequence of the randomized CI-2A gene above.

Fig. 7: Construction of a CI-2A presented library using *Bam*HI
and *Sal*I cloning site.
15 A) The 5' part of the CI-2A cDNA can be made by using overlap-
ping oligos that after annealing are extended by the use of a
DNA polymerase. The resultant fragment can then be cleaved
with *Bam*HI and *Sal*I before ligation into pCMVbipep/muCI-2A.
B) Another strategy is to use the randomized oligo in a PCR
20 together with an upstream forward primer. Again a *Bam*HI/*Sal*I
fragment can be purified an ligated into pCMVbipep/muCI-2A.

PREAMBLE TO EXAMPLES

In the following, the present invention is illustrated by way
of example wherein CI-2A is used as starting point for the
25 CellScreen™ technique adapted according to the invention so as
to allow intracellular expression of a scaffold protein inhi-
bitor which is randomly modified in the active site. This
example is non-limiting, in the sense that other suitable
protein inhibitors of enzymes could be used instead of CI-2A.
30 The skilled person can readily perform the necessary substitu-

tions and modifications necessary in order to apply the prin-
ciples exemplified below using other protein inhibitors of
enzymes as starting point.

EXAMPLE 1

5 *Materials and methods*

1-a: Construction of pCMVbipep/CI-2A
The 5788 bp hybrid plasmid pCMVbipep (the sequence of which is
set forth in SEQ ID NO: 39 and which is shown schematically in
Fig. 1 with CI-2A inserted downstream the packaging signal)
10 consists of an AKV derived retroviral insert which has been
cloned into the pUC-19 cloning vector. The retroviral insert
contains a chimeric 5' LTR, allowing expression from the
strong cytomegalovirus (CMV) promoter when transcription is
driven from the plasmid. Following integration of the vector
15 into a host genome, transcription is driven from the retrovi-
ral LTR. A versatile poly-linker is present downstream of the
packaging signal. This enables the insertion of peptide li-
braries in this position. Immediately downstream of the
polylinker is an internal ribosomal entry site (IRES), derived
20 from the encephalomyocarditis (EMC) virus (Koo et al. 1992,
figure 2A-G). This allows efficient translation from the
downstream expression cassette in a CAP independent (IRES). A
Neo marker gene is found in the downstream expression cas-
sette.

25 pCMVbipep includes a chimeric CMV and Akv promotor/enhancer.
It was constructed from the vector plasmid constructs PUT 649
(CAYLA, toulouse FRANCE) and AkvBiPep (Duch *et al.*, unpub-
lished, described below). AkvBiPep was digested with *Eco*RI and
*Asc*I and the 2779 bp fragment from position 3293 through 5670
30 to 401 was isolated and ligated to a 543 bp fragment contain-

ing the enhancer/promotor of CMV position 44 to 548. This
fragment was obtained by PCR amplifying an *Eco*RI and *Asc*I
digest of PUT 649 using the following two primers
GGTCAGGAATTCTCCGGAATTGGCTAGCCTAGAGTCCGTTACATAACT (SEQ ID NO:

5 40) and
GAGGACTGGCGCGCCGAGTGTGGGGGTTCTTACCCTTTTTATAGACCTCCCACCGTACACGCC
T (SEQ ID NO: 41). The resulting fragment was digested with
*Asc*I and ligated to an *Asc*I fragment of AkvBiPep (fragment
from position 828 to 3293).

10 AkvBiPep was constructed by digesting pBiZeo-Neo (Duch *et al.*,
Biotechniques, 1999, **26**(6): 1032-4, 1036) with *Bam*HI and *Stu*I.
The fragment from 1708 through 6054 to 1240 was ligated to a
DNA fragment made by extending with two olignucleotides. The
oligonucleotides were

15 AGATCTCCGAGGCCTGGGACCCTTCGAATTCGTTAACTGATCAACGCGTTCTAGACTACATG
GCGGCCGCGTGTTT (SEQ ID NO: 42) and
GGGGGATCCAGAGCTCGAGCTTTGAAAAACACGCGGCCGCCATG (SEQ ID NO: 43).
After extension and prior to ligation, the DNA fragment was
digested with *Bam*HI and *Stu*I. This operation removes the Zeo

20 gene in pBiZeo-neo and replaces them with a polylinker.

To construct pCMVbipep/CI-2A, 2 μg pCMVbipep was digested with
*Bam*HI/*Xho*I as described in A-5. The 5778 base pair fragment
was subsequently purified from a 0.8% agarose gel according to
A-7. A CI-2A cDNA fragment encoding amino acids 21-83 (cf. the

25 amino acid numbering in SEQ ID NO: 2) of wild type CI-2A
protein fused to an in frame methionine start codon was ampli-
fied by PCR from a pUC 19 derived plasmid containing a frag-
ment encoding the wild type CI-2A protein (cf. the nucleotide
numbering in SEQ ID NO: 1) (Generous gift from Dr. Ib G.

30 Clausen, Novo Nordic A/S, Denmark) - the cDNA fragment in-
cludes the nucleotide sequence of SEQ ID NO: 1, where nucleo-
tides 249, 252, and 279 are T, C, and T, respectively. The PCR
conditions in the 4 performed reactions were as described in

A-3 using the following primers CGGGATCCATGAAGACAGTGGCCAGAG

(SEQ ID NO: 3) and CGCTCGAGTCAGCCGACCCTGGGGACCT (SEQ ID NO: 4)

which flank the specified region of CI-2A with recognition

sites for BamHI and XhoI. The reaction mixture was applied to

5   15 three-step cycles of 94°C for 1 minute, 60°C for 30 se-

conds, 72°C for 30 seconds followed by purification of the

amplified DNA fragment as described in A-3 with a 100 µl

elution volume. The purified DNA fragment was then digested

(see section A-5) and purified (see section A-7) using 40

10   units of BamHI and XhoI with 120 µl as final reaction volume.

This fragment was ligated into the BamHI/XhoI cleaved

pCMVbipep (see section A-1) and confirmed by DNA sequencing

(section A-8) using the pCMVbipep specific primer

CTGTATCTGGCGGCTCCGTGG (SEQ ID NO: 5).

15   1-b: Construction of pmCATIREShyg

To enable transduction of non-murine cells with retroviral

vectors harvested from the Bosc packaging cell line a vector

(pmCATIREShyg) encoding the ecotropic receptor was construc-

ted. The mCAT cDNA was obtained from pJET (Albritton et al.

20   (1989) Cell **57**, pp 659-666) and inserted into the pIRESHhyg

(CloneTech) to give rise to pmCATIREShyg. This was done by

digesting pJET in 1 x EcoRI restriction enzyme buffer together

with 2 units/µl EcoRI (see section A-5). The 5' overhangs of

the EcoRI digestion were filled out using Klenow polymerase

25   according to the manufacturer's protocol (New England Bio-

labs). After incubation for 1 hour the sample was purified by

phenol/CHCl₃ extraction (see section A-4) followed by digestion

with 20 units BamHI in a reaction volume of 20 µl (see section

A-6) and purified on a 1.0% agarose gel (see section A-7).

30   This fragment was ligated into the 5699 base pair BamHI/BstXI

fragment of pIREShyg prepared as the mCAT fragment except that

the blunt ended termini were derived from the BstXI site.

After ligation and transformation, positive clones events were

isolated and confirmed by DNA sequencing (section A-9) using

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the following primers ACAGCTGGCCCTCGCAGAC (SEQ ID NO: 6),
CCCACTGCTTACTGGCTTAT (SEQ ID NO: 7), TGGGGCTGCACGTCATTTG (SEQ
ID NO: 8), TGTGCTACGGCGAGTTTGGT (SEQ ID NO: 9),
GGTTCGTGAAAGGCTCCATT (SEQ ID NO: 10), and
5 GAAATGTTCACAATTAGCCCTG (SEQ ID NO: 11).

1-c: Construction of pCMVbipepNLS/CI-2A

An oligonucleotide encoding the SV-40 large T antigen nuclear
localization signal was added in frame to the N-terminus of
the truncated CI-2A version situated in pCMVbipep/CI-2A (Fig.
10 1). The construction was performed by PCR (see section A-2)
using 25 pmol of
GAAGATCTATGGCGGCCGCACCAAAAAAGAAGAGAAAGGTAGGATCCATGAAGACAGAGT
(SEQ ID NO: 12) and CGCTCGAGTCAGCCGACCCTGGGGACCT (SEQ ID NO:
13) as primers and pCMVbipep/CI-2A as template. This reaction
15 was performed in duplicate and applied to 25 cycles consisting
of 94°C for 1 min, 45°C for 1 minute, 72°C 1 minute. The
solution was ethanol precipitated before purification of the
250 base pair DNA fragment using a 2% agarose gel (see section
A-7). The termini of the fragment were trimmed by addition of
20 20 units of BglII and XhoI and 6 µl NEBuffer 3 to a 60 µl
reaction volume (see section A-6) including 1 mg/ml bovine
serum albumine (New England Biolabs). The cleavage products
were separated on a 2% agarose gel and the 240 base pair
fragment was subsequently purified (see section A-7) with a 50
25 µl elution volume. This fragment was finally ligated into
BamHI/XhoI cleaved pCMVbipep (see construction of
pCMVbipep/CI-2A) and confirmed by DNA sequencing (see A-8).

1-d: Construction of pCMVbipepSL/CI-2A and pCMVbipepER/CI-2A

The secretoric leader (SL) was amplified by PCR using
30 pBapePuro containing the human immunoglobulin heavy chain
signal peptide (Beerli et al. (1994) J. Biol. Chem. 269,
pp23931-23936) as template and 25 pmol of
GAAGATCTATGGACTGGATCTGGCGCATCC (SEQ ID NO: 14) and

GAGGATCCAGAATGAGCGCCGGTAGCAG (SEQ ID NO: 15) as primers. The
reaction conditions were similar to those described in con-
struction of pCMVbipepNLS/CI-2A. The amplified 74 base pair
product was after phenol/CHCl$_3$ extraction (see section A-4)

5 digested with *BamHI* and *BglII* as described in section A-5
except that the reaction included 1 μl/ml bovine serum albumin
(New England Biolabs). After digestion, the 68 base pair
fragment was purified using a 3% low melting agarose gel (see
section A-6) ending up with a 30 μl volume. A *BamHI* digested

10 pCMVbipep/CI-2A vector was prepared essentially as described
for construction of pCMVbipep/CI-2A except that 10 units of
calf intestinal phosphatase (Boehringer Mannheim) were present
during the last hour of the incubation period. The purified
secretory leader containing fragment was then ligated into the

15 *BamHI* cleaved pCMVbipep/CI-2A (as described in section A-1) to
give rise to pCMVbipepSL/CI-2A. Positive clones were confirmed
by DNA sequencing (section A-8) using CTGTATCTGGCGGCTCCGTGG
(SEQ ID NO: 16) as primer.

Construction of pCMVbipepER/CI-2A (Fig. 1) was performed by

20 addition of a retention signal to the C-terminus of CI-2A in
the context of pCMVbipepSL/CI-2A. The retention signal was
added in frame with the CI-2A sequence by PCR using
CTAATCTAGACTACAGCTCGTCCTTGTAGTCCTCGAGGCCGACCCTGGGGACCTG (SEQ
ID NO: 17) and CGGGATCCATGAAGACAGAGTGGCCAGAG (SEQ ID NO: 18)

25 and pCMVbipep/CI-2A as template. The 237 base pair fragment
was digested with *XbaI* instead of *XhoI* and ligated into a
BamHI/XbaI digested pCMVbipepSL/CI-2A prepared as *BamHI/XhoI*
digested pCMVbipep. All reaction parameters were as described
for construction of pCMVbipepNLS/CI-2A.

30 <u>1-e: Construction of pCMVbipep/muCI-2A</u>
Two silent mutations were introduced into the CI-2A sequence
by a 5 step PCR procedure. The first PCR exchanged C^{192} → A
(cf. The numbering in SEQ ID NO: 1) and the product was after

purification used as reverse primer for amplification of the
CI-2A encoding region in pCMVbipep/CI-2A. This $C^{192} \rightarrow$ A mutated
fragment was used as template for introducing the second
mutation substitution of $T^{300} \rightarrow$ C (cf. The numbering in SEQ ID

5 NO: 1) and thereby leading to a PCR-product including the
previously introduced mutation. The double mutated fragment
was used as forward primer to amplify the pCMVbipep/CI-2A
defined reading frame. All the PCRs were done in duplicate and
in essence performed as described in section A-2 with 25

10 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C
for 30 seconds. The products were pooled before purification.
In the first PCR, 50 ng of pCMVbipep/CI-2A was used as tem-
plate with 50 pmol of the following primers
CCGGCCTTATTCCAAGCGGC (SEQ ID NO: 19) and

15 CTGCCGGTGGGTACAATTGTGACCATGG (SEQ ID NO: 20). The 248 base
pair product was purified using the QIAquick PCR purification
kit (see section A-3) with 50 µl as elution volume (PCR-pro-
duct 1). The second PCR consisted of 12.5 µl PCR-product 1 and
50 pmol CTGTATCTGGCGGCTCCGTGG (SEQ ID NO: 21) as primers with

20 50 ng pCMVbipep/CI-2A as template. The complete reaction was
loaded on a 2% agarose gel and 448 base pair product was
purified as described in section A-6 (PCR-product 2). This
product was then used as template in the third PCR that be-
sides the 12.5 µl PCR-product 2 consisted of 50 pmol

25 CTGTATCTGGCGGCTCCGTGG (SEQ ID NO: 22) and
CGAGTTTGTCGACAAAGAGGCGGACGCGATCGATGCGATATTCC (SEQ ID NO: 23)
leading to amplification of a 248 base pair fragment (PCR-
product-3). This fragment was purified as PCR-product 2 before
using 12.5 µl as forward primer together with 50 pmol of

30 CCGGCCTTATTCCAAGCGGC (SEQ ID NO: 24) and 50 ng pCMVbipep/CI-2A
as template. The double mutated 448 DNA fragment was after gel
purification PCR amplified using 50 pmol of
CCGGCCTTATTCCAAGCGGC (SEQ ID NO: 25) and CTGTATCTGGCGGCTCCGTGG
(SEQ ID NO: 26). The amplified double mutated fragment was

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inserted into pCMVbipep described for construction of
pCMVbipep/CI-2A.

1-f: Construction of pFab60/CI-2A and pFAB60/muCI-2A

The fragment of the CI-2A cDNA that encodes amino acids 21-83
5   of the wild type protein was inserted into the phagemid vector
pFAB60 (Johansen et al., 1995, Protein Eng. 10, pp 1063-7).
The CI-2A cDNA fragment was amplified by PCR using
pCMVbipep/CI-2A as template with 10 pmol of the primers
ATTTGCTAGCTGCACAACCAGCAATGGCACTGAAGACAGAGTGGCCAGAGTTGG (SEQ ID
10 NO: 27) and ATAAGAATGCGGCCGCGCCGACCCTGGGGACCTGGGC (SEQ ID NO:
28) in 4 reactions performed under conditions similar to those
described under construction of pCMVbipep/CI-2A. The purified
237 base pair fragment and 2 µg pFAB60 were digested in paral-
lel using 10 units NheI and NotI in 50 ml 1 x NEBuffer 4
15 including 1 µg/ml bovine serum albumin (see sections A-5 and
A-6). The digested 225 and 4655 base pair fragments were
subsequently purified using 1% agarose gel and ligated to each
other (see section A-7) and A-1, respectively. Correct
insertion was finally confirmed by DNA sequencing using
20 CACACAGGAAACTATGA (SEQ ID NO: 29) as primer (See section A-8).
An identical approach was utilized for constructing
pFAB60/muCI-2A except that pCMVbipep/muCI-2A was used as CI-2A
cDNA source.

1-g: Construction of pFab60/muCI-2A_rc

25 Substitution of amino acid 59 to 62 in the full length CI-2A
sequence with a 19-mer randomly composed amino acid sequence
was performed in the context of pCMVbipep/muCI-2A and the
modified CI-2A was subsequently moved to pFAB60 following the
same procedure as for constructing pFAB60/CI-2A. The coding
30 region for the amino acid sequence was obtained from a
synthetic oligo that was amplified by PCR. Four parallel
reactions were performed using 12.5 pmol of
CTGCCGGTGGGTACAATTGTGCTGCGCTACATGGACCGCGCAATAGTGATGAACGTGAACAT

TAGCGCACGCAAACTACGGATTGATCGCGTCCGCCTCTTTGTCGACAAACTCG (SEQ ID
NO: 30) as template with 50 pmol of the primers
CGAGTTTGTCGACAAAGAGGCGGAC (SEQ ID NO: 31) and
TCTGCCGGTGGGTACAATTG (SEQ ID NO: 32). These reactions were
5 applied to 25 cycles of 94°C for 2.5 minutes, 45°C for 2
minutes, and 72°C for 2 minutes with all other conditions as
described in section A-2. The product was purified by phe-
nol/CHCl$_3$ extraction and dissolved in 30 µl sterile H$_2$O. To
facilitate insertion into muCI-2A, 5 µl of the fragment was
10 supplied with 20 units MunI (Boehringer Mannheim), 5 µl 10 x
SureCut buffer M (Boehringer Mannheim) and sterile H$_2$0 to
adjust the volume to 50 µl, and incubated at 37°C for 4 hours.
After addition of 6 µl 10 x SalI restriction enzyme buffer and
3 µl H$_2$0 together with 20 units of SalI the incubation con-
15 tinued for 4 hours. Purification of the 89 base pair fragment
was done using a 2.5% low temperature agarose gel (see section
A-6). Preparation of the MunI/SalI digested pCMVbipep/muCI-2A
was in principle similar to the procedure described above.
Four µg pCMVbipep were digested with 30 units of MunI in a 150
20 µl reaction followed by addition of 30 units SalI in 50 µl 4 x
SalI restriction enzyme buffer and purified using a 1% gel as
described in section A-7. The ligation and confirmation
procedures were performed as for the construction of
pCMVbipep/CI-2A.

25 1-h: Library construction
The degenerated oligo
TCTGCCGGTGGGTAGAATTCNNNNKNNKNNKNNKNNKNNKNNKNNKCGGATTGATCGCGTCC
GCCTCTTTGTCGACAAACTCG (SEQ ID NO: 33) was converted into a
double stranded form by an extension reaction. The oligo was
30 mixed with a 3-fold excess of CGAGTTTGTCGACAAAGAGGCGGAC (SEQ
ID NO: 34) in 1 x SuperTaq buffer (Enzyme Technologies Ltd.)
including 8 units SuperTaq polymerase (Enzyme Technologies
Ltd.) and 0.2 mM dNTP. Heating of the mixture to 94°C for 1
minute followed by 45°C for 2 minutes was done to ensure a

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sufficient annealing between the oligos before the temperature
was changed to 55°C for 45 minutes to increase polymerase
activity. After the reaction was completed, the sample was
phenol/CHCl$_3$ extracted before cleavage of 1/3 of the product
5 with 100 units EcoRI and SalI in 100 µl 1 x EcoRI buffer
including 1 µg/ml bovine serum albumin (See section A-6). The
complete digested 56 base pair DNA fragment was purified on a
2% low temperature agarose gel (see section A-6) and ligated
(see section A-1) into the MunI/SalI cleaved pCMVbipep/muCI-2A
10 described in Example 1-g. Randomly chosen colonies were se-
quenced as described for construction of pCMVbipep/CI-2A.

1-i: Subtilisin assay
The cell extracts were diluted to the indicated amounts in 10
µl PBS and added to 25 µl 0.1 M Tris/HCl pH 8.6 containing 5 ×
15 10^{-8} M Subtilisin Carlsberg (Sigma). After incubation at 25°C
for 30 minutes, 25 µl 0.1 M Tris/HCl pH 8.6 containing 5 mM N-
succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (Sigma) was added and
the substrate conversion was followed by measuring the absor-
bency at 405 nm every two minutes until the reaction had
20 reached exhaustion.

1-j: Cell lines and culturing conditions
All the cells were cultured in Dulbeco's modified Eagle's
medium (DMEM) containing 10% fetal calf serum or 10% new born
calf serum, 1% L-glutamine, and 50 µg/ml penicillin/strep-
25 tomycin and incubated under standard cell culture conditions.

The 293mCAT cells and U2OSmCAT cells are derived from 293
(ATCC# CRL-1573) and U2OS (ATCC# HTB-96) by stable transfec-
tion with pmCAT/IRES-hyg. The 293 cells were transfected by
the Calcium co-precipitation method outlined for transfection
30 of the Bosc packaging cells and the U2OS cells were tranduced
using the Fugene transfection kit according to the manufac-
turer's protocol (Boehringer Mannheim). Stable transfectants

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were selected by culturing in the presence of 150-200 μg
active hygromycin B/ml (Sigma). After transducing 293mCAT or
U2OSmCAT with retroviral vectors the selection was shifted to
0.6 mg/ml or 0.4 mg/ml geniticin (GibcoBRL), respectively.

5 The murine NIH-3T3 cell line (ATCC# CRL-165) was upon
   transduction with the retroviral vectors cultured in the
   presence of 0.6 mg/ml geniticin.

   1-k: Transduction of NIH-3T3, 293mCAT, and U2OSmCAT cells
   For production of retroviral vector particles, pCMVbipep
10 derived constructs were transfected into the BOSC packaging
   cell line using a CaPO$_4$ co-precipitation method. BOSC packaging
   cells (also known as BOSC23 cells, cf. WO 94/19478) were
   diluted to 5 × 10^5 cells/cm^2 the day before the transfection
   and washed once in complete DMEM 2 hours before the transfec-
15 tion. The CaPO$_4$ co-precipitated mixtures were prepared by
   diluting 10 mg of pCMVbipep or the pCMVbipep derived construct
   with 10 mg salmon sperm DNA in 450 ml ddH$_2$O and adding 50 ml
   2.5 M CaCl$_2$. These solutions were slowly added to 500 ml 2 ×
   HEPES-buffered saline pH 7.05 (280 mM NaCl, 1.5 mM Na$_2$HPO$_4$, 50
20 mM HEPES/NaOH pH 7.05) under gentle shaking followed by two to
   five minutes of incubation at 25°C before adding the precipi-
   tate to the prepared BOSC cells. After 24 hours of incubation,
   the cells were washed twice in PBS (137 mM NaCl, 2.7 mM KCl,
   8.3 mM Na$_2$HPO$_4$ 1.4 mM KH$_2$PO$_4$) and further cultivated in 10 ml
25 DMEM including supplements for another 24 hours. The media
   from the transfected BOSC cells were collected and diluted
   from 10 to 10^5-fold in complete DMEM including 6 mg/ml Polybre-
   ne (Sigma). The recipient cells, which had been plated at 10^4
   cells/cm^2 and incubated at standard cell culture conditions for
30 24 hours in complete DMEM, were exposed to virus-containing
   media for 24 hours at standard conditions. After this incu-
   bation period, the transduced cell were washed twice in PBS
   and incubated in complete DMEM including geneticin.

1-l: Preparation of total cell extracts

The CMVbipep/CI-2A or CMVbipep transduced NIH-3T3 cells were
harvested from two confluent T175 cell flasks by incubation
with 5 ml 0.5 × Trypsin-EDTA solution (GibcoBRL)/plate. The

5   recovered cells were diluted 1:1 in complete DMEM and subse-
quently collected by centrifugation, washed twice in 10 ml
complete DMEM and twice in 1 ml PBS. Finally, the cells were
suspended in 100 µl PBS. The cells were rendered permeable by
three cycles of freezing in liquid nitrogen and thawing by

10 incubation at 37°C and subsequently centrifuged at 20000 × g
for 15 minutes to remove cell debris. To inactivate endogenous
protease activity, the extracts were incubated at 65°C for 15
minutes and re-centrifuged.

1-m: Preparation of nuclear extracts

15 Two approximately 80% confluent T-175 cell flasks of each cell
line were used. The cells were harvested by addition of 3 ml
Trypsin/EDTA solution (GibcoBRL) to each flask followed by
centrifugation of the 6 ml cell suspensions. All the following
reactions were performed at 4°C using chilled solutions. The

20 harvested cells were washed in 10 ml complete DMEM, 10 ml PBS,
and 2 times in 1 ml PBS. After the last wash the cells were
suspended in 1 ml NP40 lysis buffer (10 mM Tris/HCl pH 7.4, 10
mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40), mildly mixed and incu-
bated on ice for 5 minutes. The cells were then collected by

25 centrifugation at 500 x g at 4°C for 5 minutes and suspended
in 1 ml NP-40 buffer and the nuclei were immediately harvested
by repeating the centrifugation. Proteins were extracted from
the nuclei by suspension in 50 µl low salt buffer (20 mM HEPES
pH 7.9, 25% glycerol, 0.02 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA)

30 followed by addition of 50 µl High salt buffer (Low salt
buffer supplied with 1 M KCl). After 30 minutes incubation on
ice, the nuclear extract was cleared by centrifugation at
20000 x g for 30 minutes. The supernatants were stored at
-20°C.

1-n: SDS-PAGE and Western blots

Samples were supplied with ¼ volume of SDS-load buffer (NOVEX) including 1/10 volume 1 M dithiotreitol and heated to 95°C for 2 minutes before loading on a NuPage (NOVEX) 4-12% gradient

5   SDS-gel in 1 x MES buffer (NOVEX) and run at a constant current of 40 mA. The gels were equilibrated in blotting buffer (10mM CHAPS/NaOH pH 11.0, 10% Methanol, 0.5% SDS) for 5 minutes before transfer of the proteins to a 0.2 µm Obititran BA-S 83 membrane (Schleicher & Schoell) by semi dry blotting

10   for 70 minutes. The membrane was allowed to air dry before blocking 1 hour at room temperature in ECL-buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Gelatin, 0.1% NP-40). After blocking, the ECL-buffer was displaced with anti CI-2A rabbit serum diluted 1:1000 in ECL-buffer and the incu-

15   bation was continued for 1 hour. The membrane was subsequently washed 3 times, each by incubation for 20 minutes in ECL-buffer before adding 1:5000 fold diluted Horse Radish Peroxidase conjugated goat anti rabbit serum (Dako) in ECL-buffer and incubated and washed as described above. The

20   development of the signal was done using an enhanced chemoluminescent kit according to the manufacturer's protocol (Amersham-Pharmacia).

1-o: Production of anti CI-2A polyclonal antibodies, immunoprecipitation.

25   Rabbits were immunized with 100 µg full length recombinant CI-2A (generous gift from Dr. Ib G. Clausen, Novo Nordic A/S, Denmark) in complete Freunds adjuvant (Sigma) and boosted 4 times with 3 weeks intervals by injecting 50 µg CI-2A formulated in incomplete Freunds adjuvant. The anti CI-2A response

30   was detected by ELISA with recombinant CI-2A immobilized on a MaxiSorb plate (Nunc). Several dilutions of the CI-2A serum were allowed to bind and subsequently detected with a horse radish peroxidase conjugated goat anti rabbit antibody (Dako). All reactions were performed as described in example 1-p.

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Protein A containing sepharose CL-4B beads (Pharmacia Biotech) were pre-washed in dilution buffer (PBS including 0.1% Triton X-100, 0.1% bovine serum albumin) before addition of 40 µl bead volume to 100 µl of the harvested media together with 10

5   µl of the anti CI-2A antibody. The mixtures were rotating at 4°C for 1 hour followed by 3 washes in dilution buffer. The beads were suspended in 2 x SDS loading buffer and run on a SDS-PAGE before detection of CI-2A by western blot (Example 1-n).

10   1-p: ELISA

Purified pig anti rabbit polyclonal antibody (Dako) was diluted to 120 ng/ml in 0.1 M $NaHCO_3$ pH 8.5 before 50 µl aliquots were added to each well in a MaxiSorb plate (Nunc) and incubated at 4°C over night. The plate was washed 3 times in ELISA

15   wash buffer (0.01% pyrophosfate, 500 mM NaCl, 0.1% Triton X-100) and 50 µl rabbit anti CI-2A serum diluted 1:200 in binding buffer (PBS supplied with 0.5% bovine serum albumin, 0.5% Tween-20, 10% glycerol) was added to each well. After 1 hour of incubation at room temperature, the washing procedure was

20   repeated and the indicated number of phage particles was added in binding buffer. The phage particles were allowed to react for 1 hour at room temperature before washing the plate 10 times in ELISA wash buffer. Bound phage particles were detected by using a horse radish peroxidase conjugated anti

25   phage antibody (Pharmacia biotech) by binding and washing procedures as described above. The horse radish peroxidase was finally assayed using OPD-tablets according to the Manufacturer's protocol (Sigma).

Standard reactions:

30   A-1: Ligation and transformation of *Escherichia coli* by electroporation

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- 30 ng vector
- 2 µl fragment (containing a fragment concentration resulting in a 10:1 relation between fragment:vector)
- 2 µl 10 x T4 DNA ligase reaction buffer (New England
5   Biolabs)
- 300 units T4 DNA ligase (New England Biolabs)
- Sterile H_2O to adjust the volume to 20 µl
- 198 cycles consisting of 30°C for 30 seconds and 10°C for 30 minutes followed by 16°C over night

10 The ligation products were transformed into *E. Coli* by electroporation:

- 10 µl of the ligation reaction was dialyzed against 2 ml sterile H_2O
- 2 µl of the dialyzed ligation reaction were mixed with 25
15   µl competent *E. Coli* in a 0.1 cm electroporation cuvette (Bio-Rad) and pulsed with 2 kV
- Addition of 1 ml of SOC-media (20 g Bacto-Tryptone, 5 g Yeast extract, 0.5 g NaCl, 0.19 KCl, dissolved in 950 ml H_2O and adjusted to pH 7.0 using NaOH and supplied with 50
20   ml 20% glucose and $MgCl_2$ to a final concentration of 10 mM before use)
- Incubation at 37°C for 0.5-1 hours before streaking 50 µl - 200 µl on LB plates (100 ml LB (10 g Bacto-Tryptone, 5 g Yeast extract, 5 g NaCl, H_2O to 1 l) including 1.5 g
25   agar and 50 mg/ml carbenicillin)

The electroporation competent *E. Coli* cells were prepared as described below:

- An over night culture was used to inoculate 37°C LB to achieve an OD_{600} below 0.020
30 - The culture was incubated at 37°C until OD_{600} was between 0.8-1.0 and subsequently chilled on ice

- The chilled culture was harvested by centrifugation
- The harvested bacteria were suspended in 300 ml 10% ice cold glycerol solution
- Repetition of the centrifugation
5 - The suspension and centrifugation steps were repeated twice
- The bacteria were suspended in 50 ml 10% ice cold glycerol solution and recollected by centrifugation
- The bacteria were suspended in 10% ice cold glycerol
10 buffer to a final density at $6-12 \times 10^{12}$ cells/ml and stored at -80°C in aliquots

A-2: Polymerase chain reactions using TaqGold™

- 100 ng template
- 10 pmol of specified primers, unless otherwise indicated
15 - 5 µl 10 x TaqGold™ reaction buffer (Perkin Elmer)
- 3 µl $MgCl_2$ solution for TaqGold™ (Perkin Elmer)
- 0.4 µl 25 mM dNTP (dGTP, dCTP, dATP, and dTTP)
- 0.4 µl 5 units/µl TaqGold™ (Perkin Elmer)
- Sterile H_2O to 50 µl
20 - All reactions were initiated by 10 minutes at 95°C and after the specified cycles followed by 72°C for 4 minutes.
- For purification of PCR products see section A-3

A-3: PCR using Vent™ DNA polymerase

25 - 100 ng template
- 10 pmol of specified primers, unless other is indicated
- 5 µl 10 x Vent™ DNA Polymerase reaction buffer (New England Biolabs)
- 0.4 µl 25 mM dNTP
30 - 0.4 µl 5 units/µl Vent™ DNA Polymerase (New England Biolabs)

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- Sterile H$_2$0 to 50 µl
- All reactions were initiated by 1 minute at 94°C and after the specified cycle program followed by 72°C for 4 minutes

5 The amplified DNA fragments were purified by phenol/CHCl$_3$ extraction followed by ethanol precipitation if the size was less than 100 base pairs. Otherwise, the QIAquick™ PCR-purification kit was used (Qiagen). The PCR-purification kit was used according to the manufacturer's protocol with 50 µl as
10 the elution volume.

A-4: Phenol/CHCl$_3$ extraction

- The sample volume adjusted to at least 200 µl with sterile H$_2$0
- Addition of 1 volume Phenol pH 6.7 (supplier)
15 - Mixed extensively and centrifuged.
- Addition of 1 volume phenol/CHCl$_3$ 1:1 solution to the water phase
- Repeat the mix-spin procedure
- Addition of 1 volume CHCl$_3$ to the water phase
20 - Repeat mix-spin procedure
- Water phase supplied with 1/10 volume 3M NaAc pH 6.0 and 2.5 volume -20°C 96% ethanol
- Centrifuged at 20.000 x g for 20 minutes, displacing the supernatant with 70% ethanol and repeated the centrifuga-
25   tion for 2 minutes
- After the DNA was air dried it was dissolved in sterile H$_2$O

A-5: Vector preparations

The plasmid DNA used for vector preparations was purified by
30 Qiagen maxi prep columns according to the manufacturer's

protocol (Qiagen). All the used restriction enzymes and their
reaction buffers were purchased from New England Biolabs
unless otherwise indicated.

- 5 μg - 10 μg vector DNA
5 - Approximately 5 to 10 units of the indicated restriction
    enzymes pr. μg of plasmid DNA
- 1/10 of the final volume 10 x *Bam*HI restriction enzyme
    buffer unless other is indicated
- Sterile H_2O to the indicated final volume
10 - Incubation at 37°C until complete digestion was achieved
- 65°C for 20 minutes
- Purification of the digested fragment as described in A-7

A-6: Fragment preparation

As for vector preparation (see A-6) except for the amount of
15 DNA and that the purification of cleaved DNA fragments smaller
that 100 base pairs were done using low melting agarose. The
agarose slice was supplied with 300 μl 0.3 M NaAc, pH 6.0 and
incubated at 65°C until the agarose was completely melted
followed by phenol/$CHCl_3$ extraction and ethanol precipitation
20 (see A-4) to purify the DNA.

A-7: gel extraction using Qiagen gel purification kit

- Samples were added 1/6 volume DNA load buffer (60% gly-
    cerol, 0.025% Bromphenol blue, 0.025% Xylene Cyanol)
- Loaded on an agarose gel in 1 x TBE (89 mM Tris-borate,
25   89 mM Boratic acid, 2 mM EDTA)
- After a satisfactory separation was achieved the DNA was
    extracted using the Qiagen gel extraction kit according
    to the manufacturer's protocol with an elution volume of
    50 μl onless otherwise indicated

A-8: DNA sequencing

All DNA sequencing was performed with the DNA sequencing Kit
BigDye™ terminator cycle sequencing (Perkin Elmer) using a 25
cycle program consisting of 96°C for 10 seconds, 50°C for 5
5 seconds, and 60°C for 4 minutes. The amount of DNA was between
0.2 and 0.5 μg with 3.2 pmol of the indicated primers. The
BigDye™ was diluted twice according to the manufacturer's
advice. After the cycle program, the DNA was ethanol precipi-
tated and extensively washed with 70% ethanol before analyses
10 using an ABI prism 310 sequencing machine (Perkin Elmer).

EXAMPLE 2

Expression of CI-2A in mammalian cells

A potential scaffold for intracellular presentation of peptide
libraries expressed from the retroviral vectors utilized in
15 the CellScreen™ technology should not have any significant
effects on neither the retroviral replication cycle nor the
viability of the transduced mammalian cells. Furthermore, the
scaffold must retain a stable core structure to ensure a
constrained peptide presentation. In order to test whether
20 CI-2A fulfills these requirements we constructed pCMVbipep/CI-
2A (See Example 1-a and Fig. 1). The CI-2A expression con-
struct was in parallel with pCMVbipep transiently transfected
into BOSC packaging cells to produce viral particles for
transduction of NIH-3T3, 293mCAT, and U20SmCAT cells (Example
25 1-k). The titers obtained by transduction with the two viral
vectors were similar, indicating that the CI-2A expression
unit does not interfere with viral packaging and infection.

Total cell extracts were prepared from the transduced NIH-3T3,
293mCAT, and U20SmCAT cells and analysed for the presence of

CI-2A by western blot to obtain a direct proof of the CI-2A expression (Examples 1-1 and 1-n). A rabbit anti CI-2A serum raised against wild type CI-2A recognized a protein with migration properties that corresponded to that expected for
5 the CI-2A protein expressed from CMVbipep/CI-2A. Even an overexposure of the western blot did not reveal any bands with similar mobility properties in the extracts derived from pCMVbipep transduced cells thereby showing that soluble CI-2A is expressed by CMVbipep/CI-2A and tolerated by the tested
10 cell lines.

Interestingly, a CMVbipep/CI-2A specific band with a slightly lower mobility was reproducibly detected by western blots. The nature of this band is uncertain at present. The decreased mobility is consistent with a slight secondary modification
15 such as a phosphorylation, although no experimential proof exists. Other more trivial and less likely explanations could be a non-consensus start codon use or that the CI-2A stop codon is leaky thereby giving rise to an extended CI-2A protein. The inventors are currently in the process of characte-
20 rizing the expression products more detailed by N-terminal amino acid sequencing and Mass spectrometry.

A consistent constrained presentation of a peptide library in a scaffold context demands that the structure of the scaffold is stable in the given environment. Since CI-2A is naturally
25 found in the seeds of barley, the shift to the environment inside mammalian cells could influence the folding kinetics and thereby the stability of CI-2A. The protease inhibitory activity of CI-2A provides a simple method for assaying func-
tionality (Example 1-i), which reflects the amount of native
30 folded CI-2A. Increasing amounts of the total cell extracts used for the western blots were pre-incubated with subtilisin before measuring the residual protease active by addition of a chromogenic substrate (Fig. 2). The presence of 0.1-2 μl

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extract from CMVbipep/CI-2A transduced cells completely block-
ed subtilisin activity. In contrast, when increasing the
amount of the extracts from CMVbipep transduced cells to as
much as 10 μl no effects on the subtilisin activity was ob-
5   served. This result proves that the decrease in subtilisin
activity observed using extracts from CMVbipep/CI-2A trans-
duced cells is due to expression of CI-2A. Furthermore, the
observation that CI-2A extracted from mammalian cells is
functional suggests a native structure inside the cells, which
10 support that at random peptide library will be presented by
CI-2A in a constrained manner. By comparing the amount of CI-
2A containing extract required to inhibit subtilisin to a
standard curve based on purified CI-2A, a rough estimation of
the active CI-2A concentration can be obtained. This subse-
15 quently allows a calculation of the cellular concentration. By
doing so, we estimated the CI-2A content in the tested cell
lines to be in the μM range.

The combined evidence from the experiments described above
suggests that CI-2A is tolerated as an intracellularly located
20 protein in mammalian cells at a sufficient concentration to
exert a biological activity. Furthermore, the pronounced CI-2A
activity found in cell extracts indicates a native conforma-
tion enabling a constrained peptide presentation from the loop
region.

25 EXAMPLE 3

*Expression of CI-2A fusion proteins with a defined subcellular
localization*

A number of biological reactions are restricted to defined
cellular compartments. To increase the probability of select-
30 ing peptides that interfere with such types of reactions we

fused amino acid sequences to CI-2A that in other contexts
have been shown to direct the fusion protein to a defined
subcellular localization. Signals mediating localization to
either the endoplasmic reticulum or the nucleus were added to
5 test whether or not a restricted localization of CI-2A could
be achieved.

The nuclear localization signal from the SV-40 large T-antigen
was fused to the N-terminal of CI-2A thereby giving rise to
the pCMVbipepNLS/CI-2A construct (Fig. 1 and Example 1-c).
10 NIH-3T3 cells were transduced with CMVbipepNLS/CI-2A,
CMVbipep/CI-2A, and CMVbipep derived retroviral particles.
Western blotting and the subtilisin activity assay (see exam-
ple 1-i and 1-n) were subsequently used to analyze the CI-2A
content in total extracts and nuclear extracts prepared from
15 all the transduced cell lines. Neither the total extracts nor
the nuclear extracts derived from the CMVbipep transduced
cells were able to interfere with the protease activity of
subtilisin, consistent with the results described in example
2. In contrast, both the nuclear extracts and the total ex-
20 tracts derived from CMVbipepNLS/CI-2A blocked inhibiting the
protease activity of subtilisin whereas only the total extract
of CMVbipep/CI-2A exerted CI-2A activity (Fig. 3). Western
blotting revealed an equal amount of CI-2A in the total ex-
tracts from CMVbipep/CI-2A and CMVbipepNLS/CI-2A transduced
25 cells, thereby indicating a similar expression level. Consis-
tent with the activity test, the amount of CI-2A detected in
the nuclear extracts by western blotting was at least 10-fold
higher for NLS/CI-2A than for CI-2A. Thus, two independent
types of analysis support that the presence of the NLS results
30 in an increased concentration of NLS/CI-2A in the nucleus.

Endoplasmic reticulum localization requires targeting to this
compartment and subsequently an ongoing retention. To achieve
this, the pCMVbipepER/CI-2A construct contains a secretoric

leader (SL) peptide and a retention peptide fused to the N-
terminal and C-terminal of CI-2A, respectively (Fig. 1 and
Example 1-d). To be able to evaluate the functionality of the
retention signal, the pCMVbipepSL/CI-2A construct that only
5 contained the secretoric leader peptide was made (Fig. 1 and
see example 1-d). NIH-3T3 cells were transduced with these
retroviral vectors in conjunction with the CMVbipep and
CMVbipep/CI-2A. These constructs allow investigation of the
activity of both the leader peptide and the retention signal
10 by determination of the amounts of CI-2A protein secreted to
the cell media. After media exchange, the secreted CI-2A
protein was detected at different time points by a combined
immunoprecipitation western blot procedure (cf. 1-o). SL/CI-2A
was detected after 3 hours incubation and significantly in-
15 creased during the incubation periode. In contrast, ER/CI-2A
was not detectable before 5.5 hours of incubation and thereaf-
ter only at a level comparable to that produced by the
CMVbipep/CI-2A transduced cells. The CI-2A found in the media
from the CMVbipep/CI-2A and CMVbipepER/CI-2A two cell lines is
20 therefore likely to be due to cell death instead of active
secretion. In summary, the presence of the leader peptide
increased the secretion of CI-2A, but this secretion was
significantly delayed by addition of the KDEL retention sig-
nal. The combined data propose that the CI-2A expressed from
25 CMVbipepER/CI-2A becomes translocated to the endoplasmic
reticulum.

By defining the subcellular localization of a peptide library,
the likelihood of isolating active peptides interfering with
reactions known to be restricted to occur at such locations
30 can be significantly increased. In addition to the examples
described above, one could also target CI-2A to other loca-
tions such as the cell membrane, mitrocondria, lysosomes etc.

EXAMPLE 4

Displaying CI-2A on phage particles

The phage display technology has since its discovery been used
extensively for screening of peptide libraries. Phage display

5 can be used in combination with CellScreen™ to enrich the
peptide library for binders to e.g. crude cell extracts or
whole cells and thereby reduce the number of peptides needed
to be handled in the biological screening systems. We there-
fore tested the applicability of displaying CI-2A at the

10 surface of phage particles by insertion of CI-2A into the
pFAB60 phagemid (Fig. 4 and Johansen et al. (1995), Protein
Eng. 10, pp 1063-1067). The presence of CI-2A on the surface
of pFAB60/CI-2A derived phage particles was verified using
both an ELISA assay and the subtilisin assay (Example 1-o & 1-

15 i). In the ELISA assay, immobilized rabbit anti CI-2A polyclo-
nal antibodies retained phage particles derived from
pFAB60/CI-2A at levels several orders of magnitude higher than
CI-2A negative phage particles (Fig. 5). Consistent with this
result, subtilisin activity could only be inhibited by the

20 pFAB60/CI-2A derived phage particles. These two experiments
clearly demonstrate that CI-2A can be displayed on the phage
surface and therefore fulfill the features necessary for a
scaffold protein in both the phage display and the CellScreen™
technologies.

25 The loop region of CI-2A will be extensively modified in the
situation where a peptide library is presented by CI-2A. To
mimic this situation and test whether phage particles presen-
ting such modified CI-2A proteins could be produced we ex-
changed 4 amino acids situated in the loop region with a 19-

30 mer randomly composed peptide, thereby generating pFAB60/CI-
2A_rc (Fig. 4 and Example 1-g). When analyzed by ELISA, a
significant signal was obtained although it was slightly

decreased compared to the phage particles carrying the unmodi-
fied CI-2A (Fig. 5). This variation could be due the presence
of an important antibody recognition site in the loop region.
In conclusion, the fact that phage particles displaying the
5 modified CI-2A generated a signal comparable to that obtained
for the pFAB60/CI-2A containing phage particles suggests that
CI-2A can be displayed independently of the amino acid compo-
sition in the loop region.

EXAMPLE 5

10 *Constructions of CI-2A presented peptide libraries*

To facilitate the exchange of amino acids situated in the loop
of CI-2A, recognition sites for *Mun*I and *Sal*I were introduced
into the CI-2A cDNA in pCMVbipep/CI-2A by silent mutagenesis
to generate pCMVbipep/muCI-2A (Example 1-e). The presence of
15 the cleavage sites enables a non-PCR based library construc-
tion procedure. In this procedure, a synthetic oligo that
includes the randomized region is converted into a double
stranded form before cloning into the *Mun*I/*Sal*I sites (Fig.
6). The feasibility of producing a peptide library using this
20 procedure was tested by a small scale ligation followed by
sequencing of a limited number of randomly chosen clones. Out
of 8 sequenced clones, all contained insertion of the random
oligo and none of the insertions encoded identical peptides.
This suggests that that transfer of diversity from the syn-
25 thetic oligo into a biologically active form is possible using
this strategy.

The use of the *Mun*I site to generate peptide libraries limits
the portion of the loop region in CI-2A that can become sub-
stituted. To utilize the complete loop region for peptide
30 presentation it is contemplated to create a complementary

peptide library by using the more 5'-located *Bam*HI cleavage
site situated in pCMVbipep. In this case, the DNA fragment
that contains the randomized region can be generated by either
a non-PCR based or a PCR based method as outlined in Fig. 7.

5   This allows the 5'-border of the randomized region to be
defined based on theoretical considerations.

The two combined cloning strategies allow construction of CI-
2A presented libraries that diverge in the manner whereby the
peptides are presented. Such different libraries are likely to

10 complement each other regarding interactions with possible
target molecules. Screening different types of libraries will
therefore increase the number of possible target molecules
identified.

EXAMPLE 6

15   *Discussion of CI-2A as scaffold in the CellScreen™ technology*

As demonstrated in Example 2, CI-2A can be expressed in a
functional form in mammalian cells. Establishment of a func-
tional system for displaying randomized peptide sequences
using CI-2A as a scaffold is thus relatively uncomplicated to

20   envisage. In order to direct the CI-2A scaffold to different
compartments of the cell, retroviral vectors harboring diffe-
rent leader sequences have been constructed. The data pre-
sented in Example 3 illustrates that a defined localization
for CI-2A can be obtained. Especially the nucleus and the

25   endoplasmic reticulum are compartments where several specific
reactions occur, such as transcriptional regulation and recep-
tor folding. Such processes are obvious targets for peptide
antagonists. The intracellular tolerance to CI-2A in mammalian
cells and the capability of CI-2A to translocate to the nu-

30   cleus and the endoplasmic reticulum makes it reasonable to

assume that CI-2A can be targeted to other compartments and
intracellular organelles if desired.

CI-2A is an extremely stable protein that has the advantage of
being small, having no disulfide bridges, no glycosylation
5 sites and a loop of eight amino acids that protrudes from the
core structure (Macphalen C.A. et al. (1983) J. Mol. Biol.
168, pp 445-447). It has been shown that insertion of 7, 9, 11
and 13 residues between the Met40 and Glu41 (corresponding to
the Met59 and Glu60 in the native molecule) in CI-2A have a
10 minimal effect on the stability and folding rates of the
protein. Moreover, CI-2A has been found to fold through inter-
actions of key residues in the C-terminal domain of the pro-
tein, irrespective of the amino acids situated in the loop
region (Osmark P. et al. (1993) Biochem. 32, 11007-11014,
15 Ladurner A.G. and Fersht A.R. (1997) J. Mol. Biol. 273, pp
330-337). The loop therefore seems to be suitable for the
insertion of random residues which is the purpose of the
present invention. As described in example 4, substitution of
4 amino acids in the loop region with a 19-mer randomly com-
20 posed peptide did no significantly affect the capability of
this modified CI-2A to be displayed on the phage surface. This
result correlates with previous data showing that folding of
the CI-2A core structure is independent of both the size and
amino acid sequence of the loop region.

25 One important feature of the present invention is that the
peptides are selected based on a biological activity exerted
inside mammalian cells. This implicates that the stability of
the applied scaffold most be independent of disulfide bridge
formation. Since no disulfide bridges are present in the CI-2A
30 structure the stability and the folding rate of CI-2A must be
independent of the redox potential of the solvent. The extrac-
tion of active CI-2A from mammalian cells suggests that CI-2A
is capable of adopting a native structure in the intracellular

environment, which is the major demand to a CellScreen™ scaf-
fold.

The N-terminal 19 amino acid residues do not have any known
function for the folding of CI-2A (De Prat Gay G. et al.
5   (1994), Proc. Natl. Acad. Sci. 91, pp 10943-10946 and refe-
rences herein). As they might be able to perform unspecific
interactions during screening and target isolation we decided
to use the shorter 64 residue version of CI-2A. In addition,
the limited size increases the accessibility of the
10   peptide/CI-2A protein to binding pockets which might increase
the number of possible targets and thereby the likelihood for
isolation of peptide antagonists.

The ability of CI-2A to be exposed at the surface of phage
particles was clearly demonstrated by the data presented in
15   Example 4. The phage display technology allows selection of
peptides that interacts with any immobilized component(s).
This could be crude cell extracts, receptor containing mem-
branes or partly purified material containing the activity
against which a peptide antagonist is desired. At present, a
20   higher diversity can be handled by phage display due to the
difference in the physical size of a phage particle and a
mammalian cell. By reducing the peptide library to a pool
which only contains peptides that are capable of interacting
with the potential target molecules the actual diversity that
25   needs to be handled inside the cells, can be significantly
reduced

To be able to isolate the CI-2A scaffold - and thereby the
target molecule to which it binds - from selected cells, a
peptide tag will be fused to the N-terminus of the truncated
30   CI-2A. We are currently considering the following tags: His-
tag, Strep-tag or FLAG-tag. However, co-immunoprecipitation
using an anti CI-2A antibody can also be used. Alternatively

to the biochemical methods, genetic approaches such as yeast
or mammalian two- or three-hybrid systems will also be applied
to identify the targets that interacts with the selected
peptides.

CLAIMS

1. A method for identifying an *in vivo* active modulator of
activity of a target enzyme, the method comprising the steps
of

5 (a)   preparing a pool of expression vectors, each vector of
        said pool containing at least one member from a library
        of randomly modified nucleotide sequences derived from a
        parent nucleotide sequence encoding a parent peptide or
        parent ribonucleic acid which modulates the target enzyme
10   activity,

   (b)   transforming a population of substantially identical
        cells with said vectors of said pool so as to obtain
        transformed cells, said substantially identical cells
        being ones which harbour the target enzyme,

15 (c)   culturing said transformed cells under conditions
        facilitating expression of said randomly modified nucleo-
        tide sequences,

   (d)   examining said transformed cells and isolating transform-
        ed cell(s) wherein the activity of the target enzyme is
20   modulated, and

   (e)   identifying the modulator by determining said randomly
        modified nucleotide sequence of said vector present in
        cell(s) isolated in step (d) and/or determining the amino
        acid sequence or the ribonucleic acid sequence of the
25   expression product encoded by said randomly modified
        nucleotide sequence.

2. The method according to claim 1, wherein the randomly modified nucleotide sequences consist of 1) an invariable part of the parent nucleotide sequence, and 2) random nucleotides.

3. The method according to claim 2, wherein the invariable
5 part of the parent nucleotide sequence encodes a scaffold portion of the parent peptide or of the parent ribonucleic acid which serves to stabilize said polypeptide fragment or ribonucleic acid fragment.

4. A method for identifying a modulator in the form of a
10 biologically active polypeptide fragment or ribonucleic acid fragment which is capable of detectably modulating, *in vivo*, a phenotypic trait in a cell, the method comprising the steps of

(a) preparing a pool of expression vectors, each vector of said pool containing at least one member from a library
15   of randomly modified nucleotide sequences derived from a parent nucleotide sequence encoding a parent peptide or parent ribonucleic acid which *in vivo* modulates activity of a known enzyme, wherein the randomly modified nucleotide sequences comprise
20   -   an invariable part encoding a scaffold portion of the parent peptide or of the parent ribonucleic acid, said scaffold portion serving to stabilize said polypeptide fragment or ribonucleic acid fragment, and
25   -   random nucleotides,

(b) transforming a population of substantially identical cells with said vectors of said pool so as to obtain transformed cells,

(c)   culturing said transformed cells under conditions
        facilitating expression of said randomly modified nucleo-
        tide sequences,

(d)   examining said transformed cells and isolating transform-
5   ed cell(s) wherein the preselected phenotypic trait is
        modulated thereby indicating that the expression product
        of said randomly modified nucleotide sequence is biolo-
        gically active, and

(e)   identifying the modulator by determining said randomly
10   modified nucleotide sequence of said vector present in
        cell(s) isolated in step (d) and/or determining the amino
        acid sequence or the ribonucleic acid sequence of the
        expression product encoded by said randomly modified
        nucleotide sequence.

15 5. The method according to any of the preceding claims wherein
the substantially identical cells are prokaryotic cells.

6. The method according to any one of claims 1-4, wherein the
substantially identical cells are eukaryotic cells.

7. The method according to claim 6, wherein the eukaryotic
20 cells are selected from the group consisting of fungal cells,
protozoan cells, animal cells, and plant cells.

8. The method according to claim 7, wherein the animal cells
are selected from the group consisting of mammalian cells,
arthropod cells such as insect cells, avian cells, and piscine
25 cells.

9. The method according to any of the preceding claims, where-
in the transformed cells examined in step (d) predominantly
carries one single copy of the vector.

10. The method according to claim 9, wherein transformation step (b) is performed under such conditions that the cells transformed are predominantly or at most transformed with one single vector from said pool, or wherein, prior to carrying

5 out step (d), cells being transformed with more than one vector from said pool are substantially excluded from the further steps.

11. The method according to any of the preceding claims, wherein the modulator is a peptide.

10 12. The method according to any of claims 1-10, wherein the modulator is a nucleic acid fragment such as an RNA fragment.

13. The method according to any of the preceding claims, wherein the modulator is stable towards proteolytic attack and/or is insensitive to a reducing environment.

15 14. The method according to any one of claims 2-13, wherein the random nucleotides are introduced in part(s) of the parent nucleotide sequence which encode(s) the active site(s) of the parent peptide or parent ribonucleic acid, or the part(s) which encode(s) structure(s) interfering with the active

20 site(s).

15. The method according to any one of claims 2-14, wherein the invariable part of the nucleotide sequence encodes truncated parts of the parent peptide or parent ribonucleic acid sufficient to maintain stability.

25 16. The method according to any of claims 2-15, wherein the invariable part of the parent nucleotide sequence encodes a peptide which is free from disulfide bridges.

17. The method according to any one of claims 2-15, wherein the invariable part of the parent nucleotide sequence encodes a peptide having disulfide bridges.

18. The method according to any one of claims 2-17, wherein the random nucleotides are introduced in the form of an insertion or a substitution into the parent nucleotide sequence, optionally in combination with deletion(s) in the parent nucleotide sequence.

19. The method according to claim 18, wherein the number of random nucleotides which are introduced is in the range from 3 to about 100.

20. The method according to any one of claims 2-19, wherein the random nucleotides are nucleotide sequences and/or are single random nucleotides introduced at specific sites in the parent nucleotide sequence.

21. The method according to any one of claims 2-20, wherein the random nucleotides are selected from the group consisting of

- synthetic, completely random deoxyribonucleotides;

- synthetic random DNA sequences, wherein limitation on randomization of some nucleotides is introduced so as to limit the number of available sequences and/or to avoid undesired stop codons and/or to facilitate introduction of post-translational modifications of expressed peptide(s);

- synthetic random DNA sequences as in (1) or (2) coupled to a sequence encoding a purification tag; and

- CDR encoding nucleotide sequences isolated from a library
  of immune-competent cells raised against an antigen.

22. The method according to claim 21, wherein the CDR encoding
nucleotide sequences encode CDR-3 peptide sequences.

23. The method according to any one of claims 20-22, wherein
the random nucleotides are prepared by random codon synthesis
where defined DNA codons are synthesized in a random order.

24. The method according to claim 23, wherein the relative
amount of synthesized codons ensure that all encoded amino
acids will be present with substantially the same frequency in
the total of encoded polypeptide fragments.

25. The method according to any one of claims 2-24, wherein
the random nucleotides are introduced into the expression
vector by the principle of site directed PCR-mediated mutagen-
esis.

26. The method according to any one of the preceding claims,
wherein the modulator *in vivo* reduces or increases K_M of the
target enzyme for at least one substrate.

27. The method according to any of the preceding claims,
wherein the modulator *in vivo* reduces or increases V_{max} of the
target enzyme for at least one substrate.

28. The method according to any one of the preceding claims,
wherein the parent peptide or parent ribonucleic acid is an
inhibitor of activity of the target enzyme.

29. A method according to claim 28, wherein the inhibitor is
selected from the group consisting of

a BPTI/Kunitz family protease inhibitor, a serpin family
protease inhibitor, a Kazal family protease inhibitor, a
soybean trypsin inhibitor (Kunitz) family protease inhibitor,
a potato inhibitor I family member, a Bowman-Birk family
5 protease inhibitor, a squash inhibitor family member, a
wap-type 'Four-disulfide Core' proteinase inhibitor, a hirudin
family protease inhibitor, a factor Xa inhibitor, an Ascaris
trypsin inhibitor family member, a cystatin family protease
inhibitor, a calpain family cysteine protease inhibitor, a
10 tissue inhibitor of metalloproteinases family member, a
carboxypeptidase A inhibitor, a metallocarboxypeptidase
inhibitor, an angiotensin-converting enzyme inhibitor, a
cereal alpha-amylase/trypsin inhibitor family member, an
alpha-amylase/trypsin inhibitor homologous to thaumatin, an
15 alpha-amylase/subtilisin inhibitor family member, an inhibi-
tors of insect alpha-amylases, an inhibitor of mammalian
alpha-amylases derived from Streptomyces species, a trehalase
inhibitor, a polygalacturonase inhibitor, a fucosyltransferase
inhibitor, a protein kinase C inhibitor,
20 an cAMP-dependent protein kinase inhibitor, a cyclic nucleo-
tide phosphodiesterase inhibitor, a protein phosphatase
inhibitor, a TCD/MRS6 family GDP dissociation inhibitor, an
ATPase inhibitor, a phospholipase A2 inhibitory protein, a
ribonuclease inhibitor, an RNA polymerase inhibitor, a DNA-
25 entry nuclease inhibitor, and a beta-lactamase inhibitor.

30. The method according to any of the preceding claims where-
in the substantially identical cells are mammalian cells and
the vector is selected from the group consisting of a retrovi-
ral vector, a vaccinia virus vector, an adenoviral vector, an
30 adeno associated virus (AAV) vector, a herpes simplex virus
(HSV) vector, an alpha virus vector, and a semliki forest
virus vector.

31. The method according to claim 30, wherein the vector is
retroviral.

32. The method according to claim 31, wherein the retroviral
vector is derived from retrovirus selected from the group con-
5 sisting of Avian Leukosis-Sarcoma Virus (ALSV), Mammalian type
C, Mammalian type B, and Lentivirus, and optionally modified
with heterologous cis-acting elements.

33. The method according to claim 31 or 32, wherein the
retroviral vector has non-identical ends.

10 34. The method according to claim 33, wherein the non-identi-
cal ends contain non-identical promoters.

35. The method according to any one of claims 31-34, wherein
the retroviral vector contains a heterologous promoter repla-
cing the viral promoter in the 5'-LTR, such as a CMV promoter,
15 an RSV promoter, an SV-40 promoter, a TK promoter, an MT
promoter, or an inducible system such as Tet or Ecdysone.

36. The method according to any one of claims 31-35, wherein
step (a) is carried out by

1)   transfecting suitable packaging cells with vectors which
20   comprise the randomly modified nucleotide sequences and
which are integratable in virions produced by said pack-
aging cells,

2)   culturing said transfected packaging cells in a culture
medium under conditions which facilitate production by
25   the packaging cells of virions containing the randomly
modified nucleotide sequences,

3)   recovering and optionally concentrating said virions, and

4)   transducing said substantially identical cells with the
      virions.

37. The method according to claim 36, wherein the packaging
cells are selected from the group consisting of PE501, Bosc23,
5 Ψ2, GP+E86, PhoenixEco, PA317, GP+AM12, DA(ampho), Bing,
FLYA13, ProPak, CRIP, ΨAM, Phoenix-Ampho, PG13, H9 (293GPG),
and EcoPack.

38. The method according to any one of claims 31-37, wherein
the virions are pseudotyped retrovirus produced by an ecotro-
10 pic packaging cell line so as to confer broad tropism to the
virions produced thereby, or wherein an ecotropic receptor has
been introduced into the substantially identical cells so as
to allow transduction with ecotropic virions.

39. The method according to claim 38, wherein the ecotropic
15 receptor has been introduced in the substantially identical
cells by means of transduction.

40. The method according to any of the preceding claims where-
in the randomly modified nucleotide sequences are coupled to a
nucleotide sequence encoding at least one fusion partner.

20 41. The method according to claim 40, wherein the fusion
partner serves to facilitate expression and/or purifica-
tion/isolation and/or further stabilization of the expression
product.

42. The method according to claim 41, wherein the fusion
25 partner includes a purification tag such as His6 tag, myc tag,
BSP biotinylation target sequence, of BirA, flu tag, lacZ, and
GST.

43. The method according to claim 40 or 41, wherein the fusion partner is a sorting signal or a targeting sequence.

44. The method according to claim 43, wherein the sorting signal is a signal patch or a signal peptide.

5   45. The method according to claim 43 or 44, wherein the sorting signal effects export of the expressed peptide out of the cell or into the cell membrane, or, when the substantially identical cells are eukaryotic, into endoplasmic reticulum, into Golgi apparatus, into lysosomes, into secretory vesicles,
10 into mitochondria, into peroxisomes, or into the nucleus.

46. The method according to any one of the preceding claims, which further comprises the step of resolving the 3-dimensional structure of the identified modulator.

47. A method for the preparation of a replicable expression
15 vector, the method comprising the steps of identifying a modulator by the method according to any one of claims 1-46, and subsequently

    i)   isolating or synthesizing a nucleic acid sequence which
          encodes the modulator, and
20 ii)   engineering a replicable expression vector comprising an
          operon which comprises, in the 5'-3' direction and in
          operable linkage, 1) a promoter for driving expression of
          the nucleic acid sequence, 2) optionally a nucleotide
          sequence encoding a leader peptide, 3) the nucleic acid
25   sequence, and 4) optionally a termination signal.

48. A method for the preparation of a transformed cell carrying a nucleic acid sequence encoding a modulator as defined in any one of claims 1-46, the method comprising transforming a

suitable host cell with an expression vector prepared accor-
ding to claim 47.

49. A method for providing a modulator as defined in any one
of claims 1-46, the method comprising

5   I)   growing a transformed cell prepared according to the
          method of claim 48 in a culture medium under conditions
          which facilitates expression by the cell of the randomly
          modified nucleotide sequence, and

    II)   subsequently harvesting the expression product from the

10   cell and/or the culture medium, or

    Ia)   identifying the modulator according to the method of any
          one of claims 1-46, and

    Ib)   subsequently synthesizing the modulator by means of
          chemical synthesis on the basis of the sequence deter-

15   mined in step (e).

50. A method for isolating and/or identifying a target
biomolecule, the method comprising providing a modulator
according to the method of claim 49 and subsequently using the
modulator as an affinity ligand in an affinity purification

20 step so as to isolate the target biomolecule from a suitable
sample.

51. The method according to claim 50, wherein the affinity
purification step is an affinity chromatographic step, an
affinity mass spectrometry step, or a co-immunoprecipitation

25 step.

52. A method for isolating and/or identifying a target
biomolecule, the method comprising providing a peptide modula-
tor according to the method of claim 48 and subsequently using
the modulator as a probe against a cDNA library derived from

the substantially identical cells or using the modulator as
bait in a two- or three-hybrid system.

53. The method according to any of claims 50-52, wherein the
target biomolecule is a peptide or a nucleic acid.

5   54. The method according to claim 53 further comprising the
step of determining the amino acid sequence of the peptide or
determining the nucleotide sequence of the nucleic acid.

55. The method according to any of claims 50-54, further
comprising the step of resolving the 3-dimensional structure
10 of the target biomolecule.

56. A method for selecting a chemical compound as a putative
drug candidate in drug development, the method comprising the
steps of

-       assaying a library of chemical compounds for interaction
15   with a target biomolecule which has been de novo isolated
         according to the method of any one of claims 50-55, and
-       selecting compounds which interact significantly with the
         target biomolecule.

57. The method according to claim 56, wherein the library of
20 chemical compounds has been provided by chemical synthesis
    upon initial identification of the members of the library by
    structure-based or non-structure based computer drug-model-
    ling.

58. A method for the preparation of a medicinal product, the
25 method comprising the steps of

A)   selecting a chemical compound by the method according to
         claim 56 or 57,

B) performing pre-clinical tests with the chemical compound in order to assess the suitability thereof as a medicinal product,

C) entering, if the chemical compound is deemed suitable in step (B), clinical trials using the chemical compound in order to obtain market authorization for a medicinal product including the lead compound as a pharmaceutically active substance, and

D) upon grant of a market authorization, admixing the chemical compound with a pharmaceutically acceptable carrier, excipient or diluent and marketing the thus obtained medicinal product.

59. A method for developing a medicinal product, the method comprising that a modulator identified according to the method of any one of claims 1-46 serves as a lead compound in the drug development phase or wherein a target biomolecule isolated/identified according to any one of claims 50-55 serves as an interaction probe for the identification of putative drug candidates in the drug discovery phase.

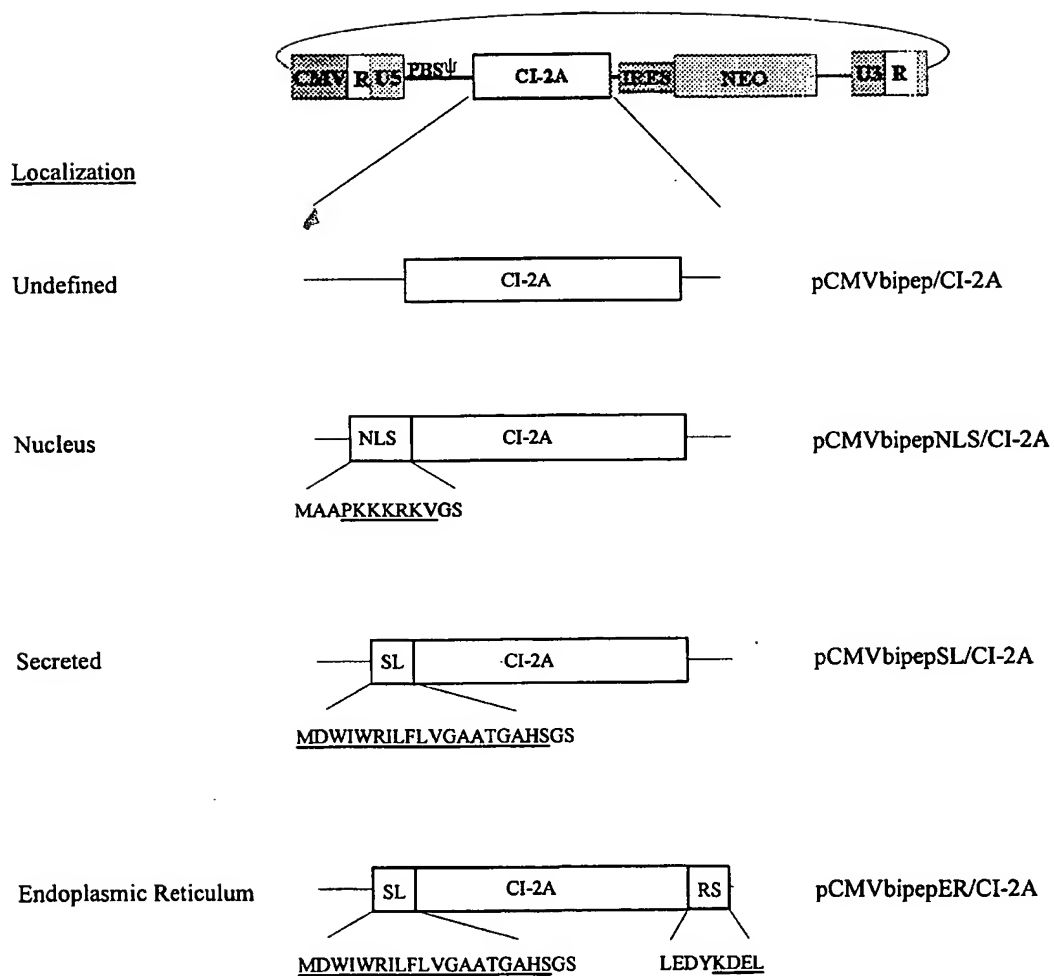Fig. 1

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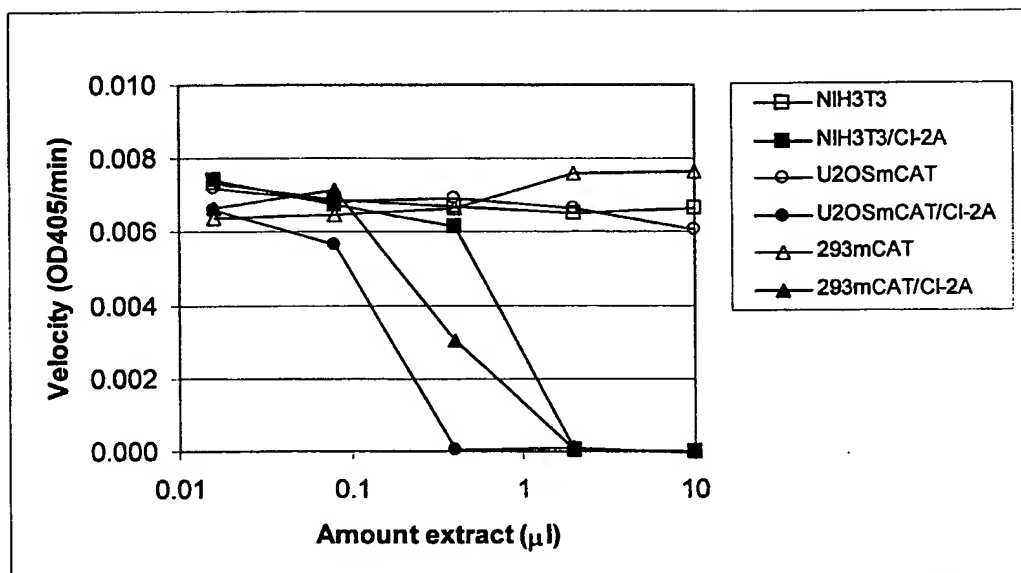
Fig. 3A

Fig. 3B

pFAB60/CI-2A

pFAB60/muCI-2A

pFAB60/muCI-2A_rc

57   62
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Fig. 4

Fig. 5

Fig. 6A

Fig. 6B

Fig. 6C

Fig. 7A

Fig. 7B

1
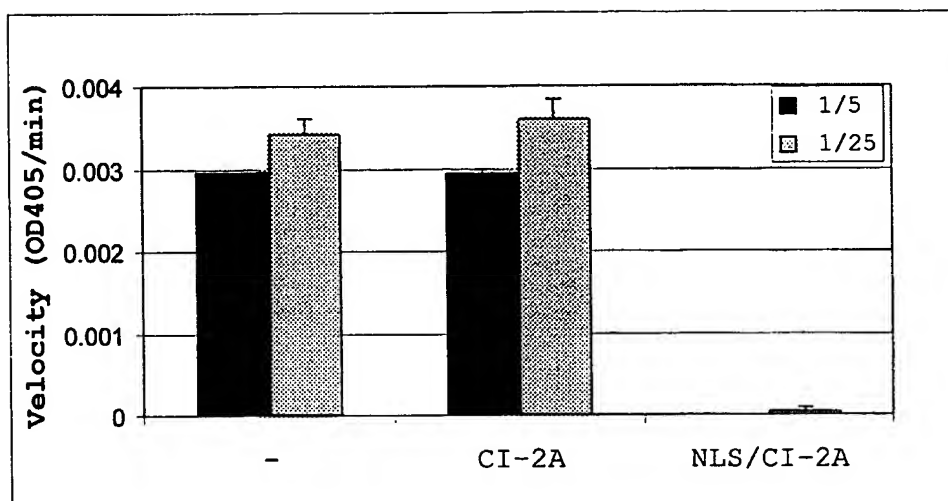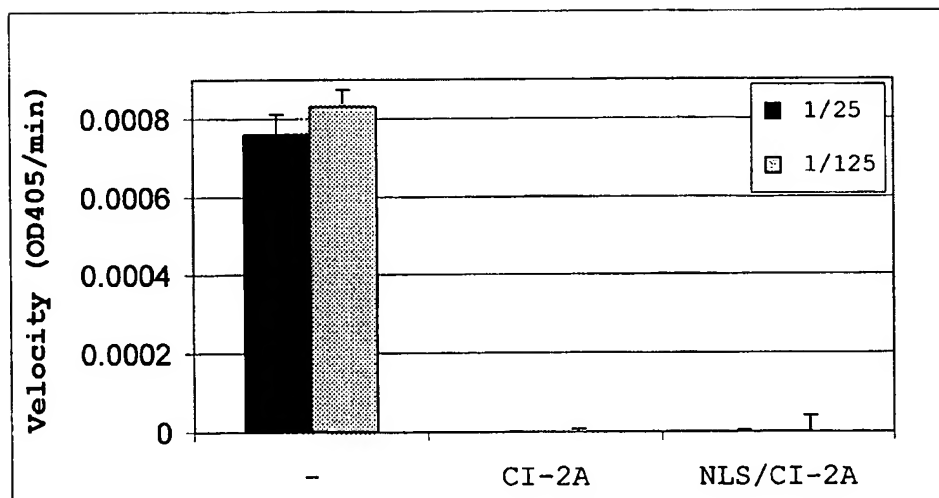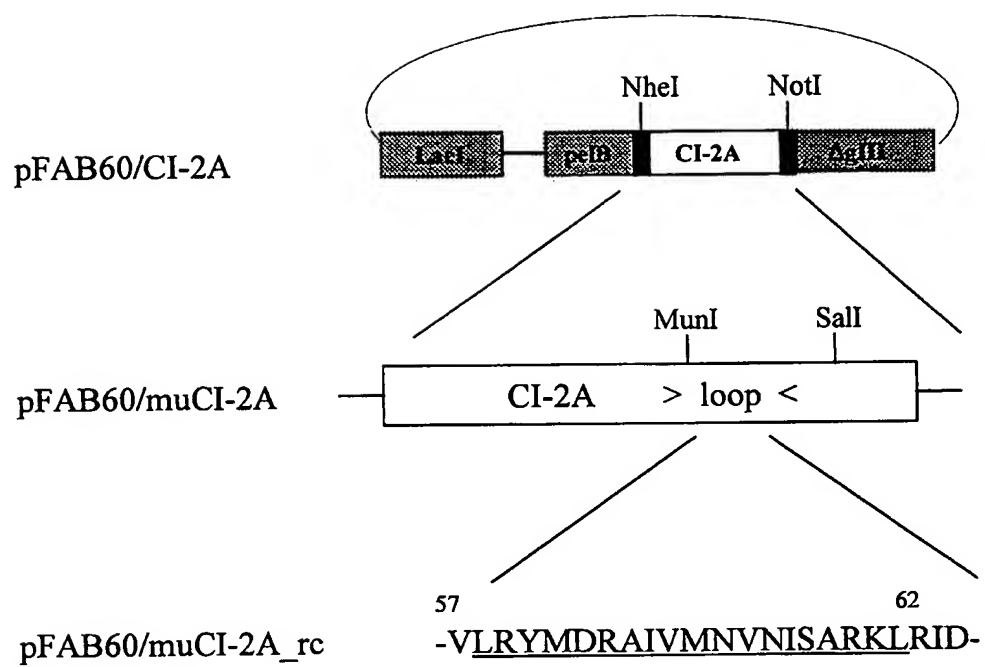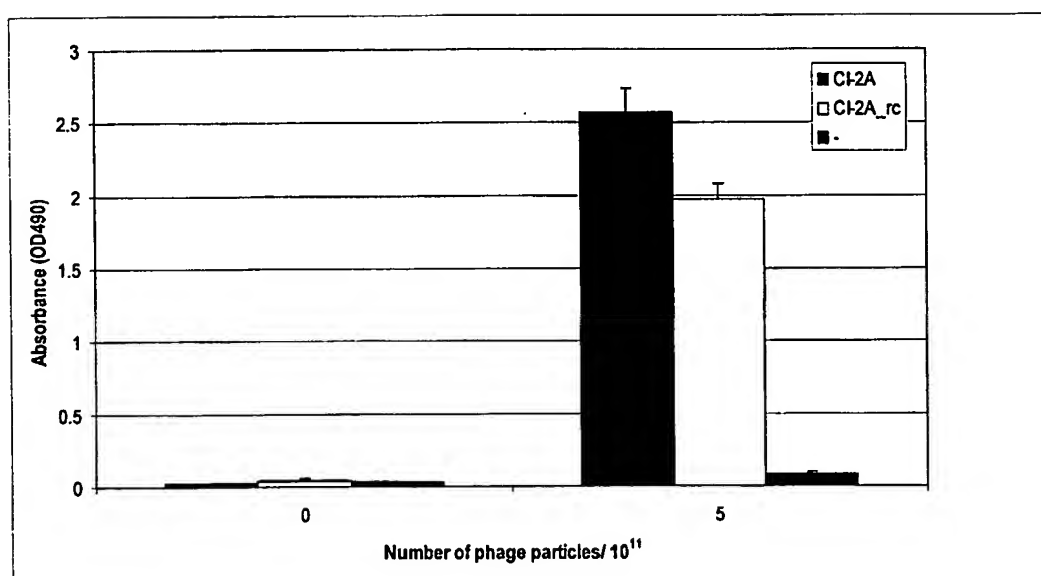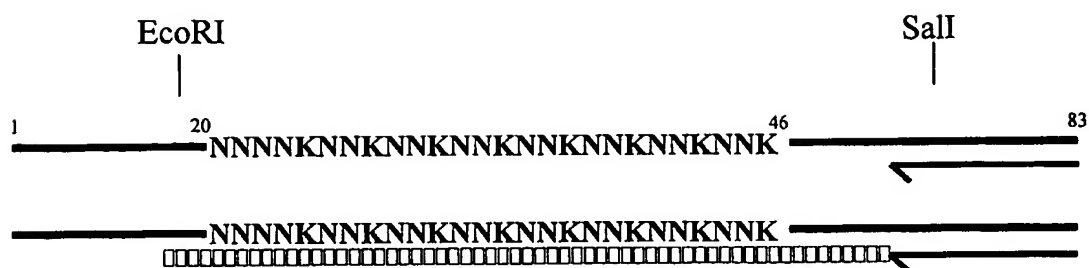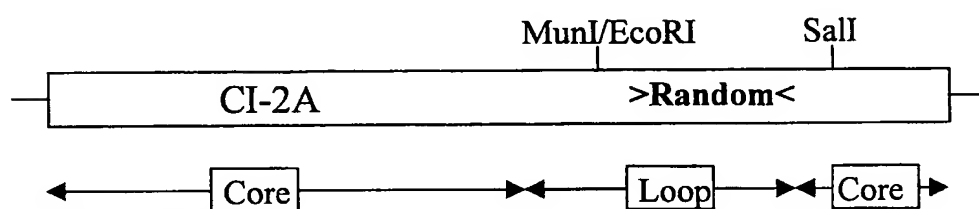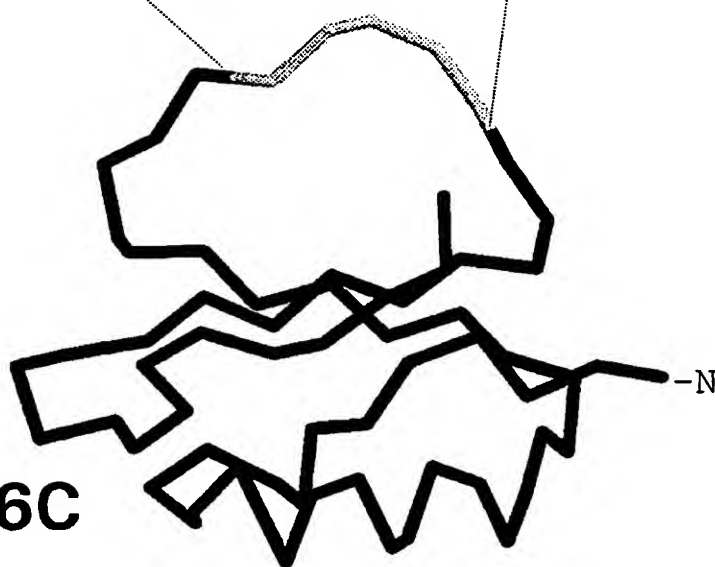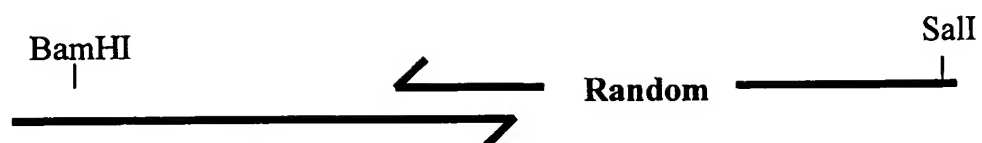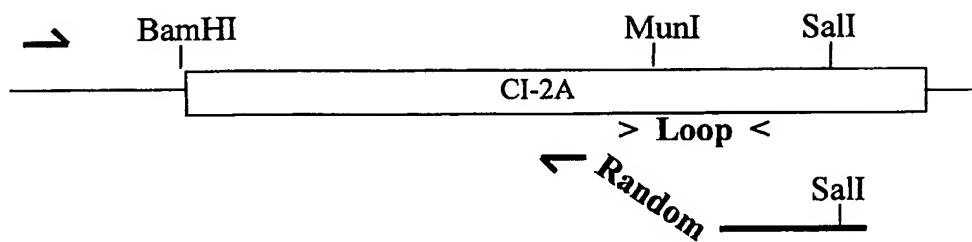
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Gly Val Asn Thr Gly Ala Gly Asp Arg His Asn Leu Lys Thr Glu Trp
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cca gag ttg gtg ggg aaa tcg gtg gag gag gcc aag aag gtg att ctg    207
Pro Glu Leu Val Gly Lys Ser Val Glu Glu Ala Lys Lys Val Ile Leu
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cag gac aag cca gag gcg caa atc ata gtt ctg ccg gtg ggg aca att    255
Gln Asp Lys Pro Glu Ala Gln Ile Ile Val Leu Pro Val Gly Thr Ile
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gtg acc atg gaa tat cgg atc gac cgc gtc cgc ctc ttt gtc gat aaa    303
Val Thr Met Glu Tyr Arg Ile Asp Arg Val Arg Leu Phe Val Asp Lys
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ctc gac aac att gcc cag gtc ccc agg gtc ggc tag caagcttgag        349
Leu Asp Asn Ile Ala Gln Val Pro Arg Val Gly
             75                  80


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         35                  40                  45

Ile Ile Val Leu Pro Val Gly Thr Ile Val Thr Met Glu Tyr Arg Ile
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<210> 10
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<210> 21
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<210> 23
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<220>
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8

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11

constituting nuclear localization signal

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 1   5

<210> 38
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 1   5   10   15

12

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13

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12Q1/68   C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12Q   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical. search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 38553 A (MOURITSEN & ELSNER A S ;JENSEN MARTIN ROLAND (DK); PEDERSEN FINN S) 5 December 1996 (1996-12-05) cited in the application the whole document	1-59
X	WO 97 27212 A (RIGEL PHARMACEUTICALS INC) 31 July 1997 (1997-07-31) see esp. claims	1-59
X	BLIND M. ET AL.,: "Cytoplasmic RNA modulators of an inside-out signal transduction cascade" PROC. NATL. ACAD. SCI. USA, vol. 96, - March 1999 (1999-03) pages 3606-3610, XP002113353 the whole document	1-59

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 1999	01/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Müller, F

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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KLUG S.J. ET AL.,: "In vitro and in vivo characterization of novel mRNA motifs that bind special elongation factor Selb" PROC. NATL. ACAD. SCI. USA, vol. 94, - June 1997 (1997-06) pages 6676-6681, XP002113354 ---	
A	FERBER M.J. ET AL.,: "Combinatorial selection of a small RNA that induces amplification of IncFII plasmids in escherichia coli" J. MOL. BIOL., vol. 279, - 1998 pages 565-576, XP002113817 ---	
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1

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			AU	5893396 A	18-12-1996
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			EP	0832207 A	01-04-1998
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			CA	2244222 A	31-07-1997
			EP	0877752 A	18-11-1998
			WO	9727213 A	31-07-1997
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